CEREAL GUMS
AND THEIR ENZYMOLYSIS

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What kind of work is that which you pursue?

Wordsworth.

CONTENTS

CEREAL GUMS AND THEIR ENZYMOLYSIS.

GENERAL INTRODUCTION.

In simple language the term modification, when applied to the malting process, means the sum total of the changes concerned in the conversion of the hard barley corn to the friable grain which we know as malt. The present investigation may therefore be regarded as an attempt to further our knowledge of certain of the biochemical changes occurring during this conversion. This is said on the understanding that considerable caution must be exercised in using the results of *in vitro* experiments to explain what happens in the grain itself. The barley corn is an extremely complex system, and types of possible action only are indicated by experiments which employ pure, natural substrates and highly purified enzyme preparations. Furthermore, the specificity of enzymes is such that still greater care is necessary in interpreting results obtained from investigations employing synthetic substrates and also from those reactions in which substrate and enzyme are obtained from different sources. The intact grain must therefore be treated with a considerable degree of respect.

Attack on any problem such as this resolves itself initially into two parts: firstly, study of the substrates involved, *i.e.* the polysaccharides themselves in this case, and secondly, study of the enzyme systems which attack them. The polysaccharides of the barley grain, other than the most abundant entity, starch, may be classified as follows:

(a) /

(a) cellulose and inert hemicelluloses,

(b) initially insoluble or labile hemicelluloses and the initially soluble hemicelluloses commonly known as the barley gums.

The present investigation concerns itself with the second group of substrates since these polysaccharides in the first group are now believed to have a purely structural function.

The earliest recorded work on the water-soluble gums is that of O'Sullivan (1882) who isolated such material from barley, wheat and rye. He referred to these products as amylans because of an assumed relationship to starch. They were obtained by water extraction of the ground grain at 40°C. after exhaustive extraction with alcohol at the same temperature. The total, crude amylan was separated into two fractions, α-amylan soluble at 40°C. but insoluble at room temperature, β-amylan soluble at room temperature. Both fractions were considered to consist solely of glucosan material of a starchy nature, an opinion later shown to be false. Then O'Sullivan extended his investigations to the malting of barley but failed to detect the highly viscous α-amylan in the finished malt.

The great importance of this work was not immediately appreciated and it was not until 1903 that investigations of a similar nature were carried out by Lindet who practised enzyme inactivation by extracting ground barley with an aqueous solution of mercuric sulphate. The resultant extract was treated with baryta and sulphuric acid to remove metallic salt and the water-soluble polysaccharides were fractionally precipitated with alcohol. A laevorotatory product of specific rotation -146°, which yielded pentoses on hydrolysis, was obtained. This apparently /

ently corresponded to O'Sullivan's β-amylan. Soon after, in 1906, the subject was taken further by Brown et al., who employed an extraction technique which involved the action of malt enzymes. The products, which constituted 6-10% of the dry weight of the barley, were not strictly comparable with those of O'Sullivan and Lindet because it is now certain that they did not represent in their entirety the original water-soluble materials of the barley. It is important to note, however, that Brown recognised that the progressive solubilisation of the barley gums was one of the most significant changes which marked the conversion of barley into malt.

There was a period of dormancy until the work of Piratzky and Wiecha (1938) who provided an immense advance in our knowledge of the barley gums. They showed that a substance with the properties of α-amylan could be obtained from the worts of undermodified malts by the addition of Fehling's solution. Subsequent purification gave a product which yielded only glucose on hydrolysis. This material appeared to be a mixture of gums of varying molecular weights of which the maximum figure recorded was 65,000. Of greater significance still is the fact that they did not detect α-amylan in malt extracts, the absence of which was anticipated by O'Sullivan.

In 1950, the work of Preece et al. went a long way towards clarifying the situation. These workers, who practised enzyme inactivation of the ground barley with 85% alcohol under reflux, isolated two water-soluble polysaccharides which they described as barley gums B_2 and C_2. They were so called according to the solubility relationships employed in the hemicellulose fractionation procedure of Norris and Preece (1930). The B_2 and C_2 fractions /

fractions corresponded respectively to the α- and β-amylans of O'Sullivan and, on analysis, B_2 was found to consist mainly of glucosan accompanied by 5-10% pentosan while C_2 contained, in all, 40% pentosan and possessed a molecular weight in the region of 50,000.

Meredith et al. (1951) also reported the isolation of gums from barley, malt and wort. However, the products are not strictly comparable because preliminary enzyme inactivation was not practised. As a consequence of this, the yields of gums were greater than those obtained by Preece et al. (loc. cit.) but it is significant, as will be observed later, that the monosaccharides, detected after hydrolysis of the products, were the same (with the addition of mannose, galactose, galacturonic acid and, possibly, ribose). It was not until 1952 (Anderson) that the Winnipeg school recognised the importance of enzyme inactivation prior to aqueous extraction of the grain. They discovered that omission of this step rendered the gums unstable in solution, the result of the action of enzymes co-precipitated with the gums.

Preece and Mackenzie (1952) then introduced what is, so far, the most detailed fractionation of the gums. The enzymes of the ground barley were inactivated according to Preece et al. (1950), then the dried grains were extracted with water at 40°C. Remarkable progress was made, in that homogeneous or near homogeneous substrates were obtained by the addition of graded quantities of ammonium sulphate to the water-bright, aqueous extracts. They found that it was possible, with repeated re-solution and re-precipitation, to obtain a laevorotatory glucosan (specific rotation -12°) free from pentosan units up to 30% ammonium sulphate concentration. This barley β-glucosan was the subject of structural /

tural investigations by Aspinall and Telfer (1954) who concluded that approximately equal numbers of -1,3 and -1,4 β-linkages were present, the molecules being of the straight-chain type. There is evidence that O'Sullivan's α-amylan and the B_2 fraction of Preece et al. (1950) are composed principally of this glucosan. It is interesting, therefore, in view of the failure by O'Sullivan and Piratzky et al. (loc. cit.) to isolate α-amylan from wort, to note that Preece and Mackenzie (loc. cit.) report the virtual absence of β - glucosan from this source.

Meredith et al. (1953) then reported the isolation of water-soluble gums from barley and malt by various procedures. In each instance preliminary refluxing of the ground grain with ethanol was employed. The dried grains were then extracted by a 1% aqueous solution of papain to give, in the case of barley, a product which they believed to be the naturally occurring, undegraded, water-soluble polysaccharide. Products of a similar composition but of lower degree of polymerisation (as indicated by the viscosities of their aqueous solutions) were obtained by extraction of ground grain with aqueous solutions of hydrochloric acid (pH 1.0) and 6% (w/v) trichloroacetic acid (pH 1.0). Later the same workers (1955) described similar methods but employed a 0.025% aqueous solution of papain. However, there is still the impression that the product obtained by papain treatment represents a greatly degraded product, the result of incomplete inactivation of enzymes. The above procedure was also adopted by Djurtoft and Rasmussen (1955) with the addition that the papain was first activated by sodium sulphide and sodium cyanide. These methods were criticised by Preece (1955) who stated that there is the possibility that the papain may contain enzymes capable of attacking hemicelluloses and furthermore the

apparent /

apparent effectiveness of the papain could be due not so much to its inhibition of degradation as to its causing aggregation of gum molecules.

The cell-wall materials which are present in an initially-water-insoluble form, the labile hemicelluloses, must be considered in parallel with the gums. The term hemicellulose was first put forward by Schulze (1891) to denote a group of substances entering into the composition of the plant cell-walls. The preparation of such materials was effected by extraction of the source material with 4% soda followed by the addition of dilute mineral acids. He considered these substances to be intermediate precursors of cellulose, but later developments have shown that there is no foundation for this belief. The hemicelluloses differ in being soluble in dilute alkali and yield primarily pentoses, but also hexoses and uronic acids, on hydrolysis with dilute mineral acid. Therefore, the term hemicellulose is rather unfortunate. Investigations of these labile hemicelluloses, which are now thought to have both a structural and a reserve function, have been hindered by their close association in the cell-walls of the endosperm with cellulose, lignin and pectic substances.

The first hemicellulose preparation is accredited to Tollens and Stone (1888) who obtained it from brewers' spent grains and which, on hydrolysis, yielded arabinose and xylose. The same two pentoses were detected by Schulze (1892) in the hydrolysates of preparations from wheat and rye. These preparations were obtained by extraction of the source material with 4% soda followed by acidification of the extract with dilute mineral acids. In the same year Schulze and Tollens prepared a xylan after washing spent grains with dilute ammonia. Preece (1931), also working with

spent /

spent grains, isolated three principal products namely xylan, urono-xylan and urono-araban which were in no way dissimilar to the hemicelluloses obtained from some beechwoods by O'Dwyer (1923) who established the presence of uronic acids in such materials. It is significant that, at this time, there appears to have been no examination of the hemicelluloses of the barley grain itself. However, as a result of the methods employed by Brown et al. (loc. cit.) their so-called gums are now considered to have been largely contaminated by hemicellulose material, the result of the solubilising action of the malt enzymes.

A major advance in hemicellulose fractionation technique was made by Norris and Preece (1930) who employed wheat bran as the source material. Pectic substances were removed by ammonium oxalate solution followed by treatment with alcoholic soda to minimise the lignin content of the products. Acidification of the alkaline extract of the bran residue yielded a polysaccharide fraction A, while two further fractions B and C were obtained from the acid solution at the respective acetone concentrations of approximately 30% and 60% by volume. Each of these fractions was subjected to further fractionation, from alkaline solution, with Fehling's solution whence A_1 and B_1 were recovered in the form of their copper complexes. A_2, B_2 and C_2 were obtained from the respective mother liquors, also as their copper complexes, by acetone precipitation. Liberation of the polysaccharide by acidification followed by examination of each, indicated that, with the exception of B_2 which appeared to consist substantially of glucosan material, they consisted of either pentosans or uronopentosans. In this manner they were able to achieve a wider fractionation of hemicelluloses than had hitherto been possible.

Criticism /

Criticism was brought to bear on this method by Norman (1937), particularly with respect to the initial alcoholic soda treatment of the tissue. It was claimed that this procedure is not without effect upon the hemicelluloses themselves especially when high temperatures are employed for prolonged periods. This claim was recently upheld by Preece et al. (1950). A further criticism points out that such a method of fractionation is based merely on solubility relationships and that there is no sound case for a purely chemical classification. This is a particularly unfair objection when it is remembered that even at the present time only the most obviously homogeneous preparations can be attacked by the newer methylation techniques for success to be possible.

However, the Norris and Preece scheme was not without effect on the subsequent study of the cereal gums by Preece et al. (1950). More recently Preece et al. (1953a and b), employing autolysis techniques, obtained evidence of an indirect nature as to the behaviour of the initially insoluble hemicelluloses in a number of barley varieties during the early stages of growth. In 1954, Preece and Hobkirk distinguished between two types of cereal grain hemicelluloses, extractable by 4% soda, (i) a husk type which is distributed throughout the grain and (ii) an endospermic or reserve type which is found in largest quantities in the inner endosperm. The husk type hemicellulose gave solutions of low viscosity and high pentosan content whereas the endospermic type gave solutions of high viscosity and exceptionally high glucosan content. Again, these workers found that all the evidence pointed to the fact that the endospermic type hemicellulose acted as a gum precursor during the early stages of growth or during autolysis.

Recently a very significant approach to the problem of the maltability /

maltability of any particular barley sample has been described by Massart and van Sumere (1955). They have based their method on the fact that the solubilisation of hemicelluloses plays a very important part in the modification of barley.

The observation by Brown and Morris (1890) of the degradation of the barley endosperm cell-walls during germination was the first indication that such mechanisms existed. This dissolution of the cell-walls, which resulted in a considerable softening of the tissues, was supported by Grüss (1902) and Ling (1904) who were able to show that the cell-wall was not entirely dissolved away, a transparent skeleton, revealed only by staining, remaining intact. Again, Hopkins and Krause (1937) stated that one or more "hemicellulases or cytases" attack the cell-walls of the endosperm, leaving a skeleton which is permeable to starch-splitting enzymes; thus breakdown of the cell-walls is envisaged as an important preliminary change which has the effect of facilitating amylolysis, proteolysis, etc. However, at that time, this action was not related to the amylans of O'Sullivan or the products obtained by Lindet (loc. cit.).

It was not until 1929 that it was realised there existed a relationship between the initially soluble and the initially insoluble hemicelluloses, when Norman pointed out that there is no essential difference, in terms of components, between the gums and hemicelluloses, but he did attribute the physical difference of solubility to aggregation and the lengths of molecular chains built up from these components. However, it should be noted that the "gums" of Norman are substances quite distinct from the cereal "gums" which figure so prominently in the present work. The relationship was put on a firm basis as a result of the work of

Preece /

Preece et al. (1950) who were, to some extent, reproducing the work of Brown et al. (1906). The former group found that upon prolonged contact between the barley gum C_2 and barley enzymes a point was reached when attempted precipitation, by the normal means, failed. However, they did obtain a similar water-soluble polysaccharide from malt, hemicellulose dextrin C_2, and this indirect evidence, together with the increased yield of this product, was taken as an indication that the malt fraction was formed, at least in part, other than from the barley gum. It was assumed that the C_2 malt fraction was formed as a result of the solubilising action of a "cytolytic" system of enzymes upon the initially insoluble hemicelluloses. The relationship between this malt gum and hemicellulose was supported further by the presence, in both, of anhydrouronic acid. This was followed by the work of Preece and Ashworth (1950) who confirmed the opinion that the initially insoluble hemicelluloses, which are not chemically dissimilar to barley gums, particularly C_2, are rendered soluble during malting.

In view of such investigations Preece (1952) came to criticise such a classification of initially insoluble hemicelluloses and water-soluble gums as artificial, and he pointed out that this tended to stress the relatively unimportant solubility character whilst obscuring the far more significant chemical resemblances between the materials. Preece then declared that the change from a water-insoluble form to a water-soluble form is an enzymic one under natural conditions but which can be likened to the action of dilute alkali. Furthermore, just as dilute alkali achieves a measure of solubilisation in the more typical hemicelluloses of woody tissue, e.g. freeing them from the supposed association with other materials in the cell-wall, e.g. lignin, so do enzyme systems attack /

attack the more intimately held hemicelluloses of the grains themselves bringing a proportion of these into a soluble form. It is therefore now convenient to refer to the initially insoluble and initially soluble entities as hemicellulosic materials. Under natural conditions Preece (loc. cit.) considers the most interesting change involved in solubilisation to be that of disaggregation which is accompanied by liberation from union with lignin. Disaggregation produces molecules of smaller chain length without any major liberation of reducing sugars or oligosaccharides. This is true, in part, but more recent evidence suggests that other factors are involved.

There exists considerable confusion in the literature regarding the nomenclature of enzymes or enzyme systems associated with the degradation of cell-wall materials. The terms range from the "cyto-hydrolytic" enzymes of Brown and Morris (1890) to the abbreviation "cytase" which is very commonly employed. Many workers have been more specific, preferring to name the enzymes according to the particular substrate which they attack, e.g. cellulase, hemicellulase, xylanase, etc. However, a more desirable nomenclature was suggested by Preece and Ashworth (loc. cit.) who distinguished between two types of enzyme systems involved in the degradation of cell-wall material, (a) a cytoclastic system of enzymes which effects a rapid decrease in molecular complexity of the substrate with little production of reducing groups (responsible for the above disaggregating effect) and (b) a cytolytic system which is characterised by the prolonged production of reducing groups. Then Preece, Aitken and Dick (1954) adopted the nomenclature being used at the present time. They referred to the cytoclastic system as endo-β-glucosanase and the cytolytic system /

system was taken to represent the combined actions of endo- and exo-β-glucosanases, together with a cellobiase system.

Investigations into such systems were hampered by the failure to isolate a homogeneous substrate. It was in 1928 that Lüers and Volkämer studied the action of extracts of green malt on xylan preparations from elder-pith and barley. They obtained evidence for the presence of an enzyme system capable of hydrolysing these substrates, the enzymic activity being estimated by measuring the increase in reducing groups at the optimum conditions for reaction, namely, pH 5.0 and 45°C. This "xylanase" system they claimed to purify by an adsorption technique (phosphate buffer, pH 5.0) using alumina as adsorbent and eluting with a phosphate buffer, pH 8.3. In 1931 Ziese reported the first investigation which employed an unnatural substrate, hydroxyethylcellulose, to demonstrate the presence of "cellulase" in barley malt.

This was followed by the work of Preece and Ashworth (loc. cit.) which could be regarded as the beginning of the modern era of enzymic investigations in this field. These workers found the mixed barley gum C_2 (approximately 40% pentosan) to be the most suitable substrate, and in this manner useful general principles were established but it was realised that with a mixed substrate no single action could be followed with any certainty. Work of a similar nature was reported by Bass et al. (1952, 1953), but the absence of a homogeneous substrate was again a great hindrance. Sandegren et al. (1952, 1953) attempted to overcome this difficulty by employing the artificial substrates, ethylhydroxyethylcellulose and carboxymethylcellulose, and they estimated the activities of their "cellulase" preparations by measuring the rate of viscosity diminution. However, the use of such substrates cannot be recommended /

recommended since considerable difficulty arises in relating the results obtained to the degradation of non-starchy polysaccharides in the intact barley grain during malting. Furthermore, it has been shown by Preece and Aitken (1953), Anderson (1955), van Roey and Hupé (1955) and Thomas (1956) that, in experiments of such a nature, the results obtained do not parallel those of investigations in which the β-glucosan of Preece and Mackenzie (1952) is employed, because the artificial substrates are found to be much less sensitive to enzyme attack. Work was therefore continued by Preece et al. (1953, 1954), Bass et al. (1955, 1956) and Meredith et al. (1955) with the substrate β-glucosan, when useful information concerning the enzymolysis of this cell-wall material was obtained.

Investigations into the pentosanase systems have not developed in parallel. The stumbling block is the common one, namely, the failure to obtain a homogeneous pentosan substrate from barley. Preece and Hobkirk (1953) have described the isolation of a substantially glucosan-free pentosan from rye, and this has been employed in investigations of the barley pentosanases by Preece and Hobkirk (1955). Such "mixed-source" investigations have their value in indicating possible modes of behaviour, but the specificity of enzymes is such that great care is essential in interpreting the results.

SCOPE OF INVESTIGATION.

It is quite clear that *in vitro* experiments with pure substrates and highly purified enzyme preparations can do no more than indicate types of possible action. Therefore, already having a method of isolating a barley β-glucosan in a very high degree of purity, means for obtaining enzyme preparations of enhanced purity were sought, and it was hoped, by employing these preparations in the degradation of initially-water-soluble β-glucosan, to provide further evidence of the complex nature of the degradative scheme. Included in this approach are attempts to differentially inactivate endo- and exo-β-glucosanases and cellobiase, and it will be seen that confirmation of an earlier assumption of the mechanism for the degradation of β-glucosan has arisen from this study. Furthermore, chromatographic and ionophoretic analyses of the degradation products of β-glucosan enzymolysis also offer the possibility of determining the manner in which the β-1,3- and β-1,4- linkages are distributed within the β-glucosan molecule.

With the realisation of the important part played by the β-glucosanases in modification, the opportunity has been taken to show some aspects of the behaviour of endo- and exo-β-glucosanases and cellobiase during the course of commercial maltings. During one such series of investigations the water-soluble and alkali-soluble carbohydrates were isolated and analysed with the intention of obtaining some indication of the manner in which endospermic cell-walls are broken down. At the same time there was the possibility that an opportunity would arise to support or overthrow the theories that either "cellulase" activity of a barley or its green malt or gum content alone or a combination of both gives an indication of the malting properties of a particular barley.

Although /

Although the main argument is confined to enzymes of raw and germinating barley, the opportunity has been taken to provide some comparisons with the enzymes of other raw cereals.

SECTION 1.

The Malting of Barley: Enzymolysis of β-glucosan by the β-glucosanase System.

INTRODUCTION.

Cytolysis during malting is evidenced chemically by the increase in the proportions of water-soluble pentosan and glucosan materials present in the barley grain, this increase being associated with the change in the physical nature of the kernel. As has already been noted, Preece and Ashworth (1950) came to the conclusion that two types of action, cytoclastic and cytolytic, are involved, but the nomenclature of Preece, Aitken and Dick (1954) will be employed in the present investigation, which is concerned with the enzymolysis of β-glucosan.

Important initial studies of the enzymolysis of cell-wall materials were carried out by Brown and Morris (1890), Grüss (1902) and Ling (1904), whilst Brown and Morris demonstrated also the destruction of the "cytase" activity of green-malt extracts by heating at 60°C., the amylase activity surviving this treatment even at 70°C. Brown and Escombe (1898) observed that enzymic breakdown of starch granules never takes place as long as the walls of the cells containing them are intact. This is all the more remarkable in that a similar suggestion was made more recently by Dickson and Shands (1941) who showed that, during malting, the cell-walls of the endosperm are progressively dissolved from the germ portion to the distal portion of the kernel. This suggests that the embryo is constantly secreting "cytases" which diffuse towards the distal region. Amylolysis was observed only after breakdown of the cell-walls, and even then it merely occurred in those regions immediately adjacent to the embryo. Hopkins and

Krause /

Krause (1937) had reached a similar conclusion.

In 1904 Adrian Brown also investigated cell-wall dissolution by soaking thin sections of barley endosperm in extracts of green malt or oats and this was followed by the work of Horace Brown et. al. (1906). Baker and Hulton (1917) made the important observation that, by mashing together barley and green malt, more pentosan was extracted than the sum of that in separate extracts; cytolysis was indicated again, and it was shown that the active enzyme could be precipitated from extracts by treatment with alcohol.

Many years later Lüers and Volkämer (1928) studied the enzymolysis of xylan preparations by malt extracts and it was found that activity was lost at pH 9.0. In 1938 Voss and Butter carried out work of an essentially similar nature. Lüers and Malsch (1929), employing similar substrates, demonstrated, during malting, a decrease in "cytase" activity on steeping, an increase following germination and a large decrease during kilning which gave a final malt with less activity than the original barley. This may be explained by the fact that Lüers and Volkämer (loc. cit.) had already shown the inactivation of "xylanase" by heating at 60°C.

Ziese (1931), employing water-soluble cellulose derivatives as substrates, illustrated the presence of "cytase" in barley and malt, and also showed that malt "cellulase", unlike bacterial "cellulases", was not inhibited under the combined influence of copper sulphate and hydrogen cyanide.

In 1938 Piratzky and Wiecha furnished an important contribution to our knowledge of enzyme changes in the malting process. These workers employed aqueous solutions of the highly viscous α-amylan to examine the enzyme potentialities of extracts of the grain at various stages of malting and observed that green-malt extracts /

extracts decreased the solution viscosity 2-3 times as quickly as extracts of raw barley. It was found that hydrolysis was exceedingly slow even with the malt extracts and further loss of activity occurred in the kilning process.

Preece and Ashworth (1950) reported that the cytolytic system was first formed only after steeping and growth of the barley corn. However, it was apparent at that time that profitable, detailed investigations of this postulate would be impossible until unmixed substrates had been made available, and the preparation by Preece and Mackenzie (1952) of a β-glucosan, uncontaminated by pentosan, accordingly represented a turning point in this work.

The close connection between the physical changes taking place during malting and the enzymes present in the grain in amounts sufficient to degrade non-starchy polysaccharides rapidly, was then studied with a view to predicting the malting quality of a barley from the "cellulase" activity. Lundin (1951) has quoted results of this nature which seemed to indicate that barleys with low "cellulase" activities had poor germination characteristics while those with high "cellulase" activities germinated well. However, further complications arise during germination since cytolytic systems develop in the grain and, although a defined substrate is used, e.g. β-glucosan, it by no means follows that reactions with barley and green-malt extracts are the same. Preece (1951) has pointed out the probability that true cytolysis is slight during malting, and he accredits the loss of precipitability of the gums to the continued action of the cytoclastic system. The wisdom of the above method for assessing malting quality is suspect because it is difficult to see how the information which is made available from the β-glucosanase activity of barley /

barley can be used to predict the potentialities for enzyme production or activation during and subsequent to steeping.

It is therefore unlikely that measurement of enzyme activity alone will provide a sufficient basis for deducing how a particular barley may be expected to behave during growth. Of greater value would be figures combining an expression of both substrate level and enzyme potentialities. An autolysis technique which furnishes such information has been employed by Preece and Mackenzie (1953), Preece and Aitken (1954) and van Roey and Hupé (1955) with some success, but one main disadvantage is that the method can of its very nature take no account of the potentialities of the grain for producing further enzymes during the periods of steeping and growth.

Amongst other carbohydrases van Sumere and Massart (1952) have shown the presence, in raw and germinating barley, of an "hemicellulase" which attacks caroubin, a galactomannan obtained from the endosperm of caroube seeds (St. John's bread), while the presence of a laminarinase from the same sources has been demonstrated by Dillon and O'Colla (1951) as a result of the degradation of laminarin, a β-1,3-polyglucoside obtained from Laminaria digitata. If these are definite enzyme systems, their function in barley remains to be determined.

Enebo and Sandegren (1952) employed an artificial substrate, ethylhydroxyethylcellulose, in investigations of enzyme behaviour during malting. They showed that, with extracts of green malt and malt, practically all the "cellulase" activity had been destroyed by heating at 60°C. whereas with barley extracts more than 25% of the activity remained after this treatment. Unlike malt "cellulase", the barley system was not fully inactivated by treatment /

ment at temperatures above 70°C.

Continuing their work on enzyme changes in the malting process, Enebo et al. (1953) found that "cellulase" activity, measured by observing the decrease in viscosity of solutions of ethylhydroxyethylcellulose, increased remarkably and no decline could be observed even after thirteen days' growth. They also purified the green-malt system of enzymes by precipitation with ammonium sulphate and then compared the influence of added sugars on the activities of both the crude extract and the purified green-malt preparation. It was observed that cellobiose in concentrations above 1% decreased the activity of the crude extract but not the "cellulase" activity of the purified preparation which was inactivated by lactose (β-galactosidic). In the same way addition of glucose, mannose or xylose in concentrations up to 1% stimulated the activity of the purified "cellulase" preparation but had no effect when added to green-malt extract. These effects they explained on the assumption that green-malt extracts already contained such amounts of "cellulase-activating" sugars and cellobiose that no effect could be obtained by further addition of such sugars. They postulated the presence of two enzymes in green malt to explain the mechanism of activation of purified green-malt "cellulase" by glucose. One of these enzymes they took to be a typical hydrolytic β-polyglycosidase and the other a non-hydrolytic transglycosidase which, for its action, is dependent on the presence of sugars such as glucose, arabinose and xylose.

Support for this theory may be obtained from the work of Preece and Hobkirk (1955) who, using a rye araboxylan as substrate, gave reason to suppose that wheat enzymes could effect transarabinosylation. This is a very attractive explanation of the failure to detect /

detect free pentoses in malt, although they can certainly be detected earlier in the malting process. However, there seems to be no prior suggestion of the transfer of glucosyl residues with β-glucosan as substrate and with enzymes derived from barley. More evidence is certainly necessary, but the possibility of a transglycosidase, acting on the barley β-polysaccharide during germination, cannot be ignored.

Of the work done elsewhere, that of the Winnipeg Laboratory is of particular significance. Thus, Bass, Meredith and Anderson (1952, 1953), working with mixed barley gums and with enzyme preparations from barley and green malt, found evidence consistent with the view that the β-glucosanase system (previously referred to as the "cellulase" system) of green malt contained both endo- and exo-enzymes, and whilst the presence of the viscosity-decreasing factor was undoubted, it was not clear whether barley yielded the exo-enzyme. At the same time they received results which indicated that the green-malt system also contained a pentosanase. Later, Meredith and Anderson (1955), again using mixed substrates, reaffirmed the two-fold nature of gum-degradation involving endo- and exo-enzyme systems, whilst Bass and Meredith (1955) subsequently reported the action of green-malt enzymes on β-glucosan.

These latter workers concluded that two enzymes are involved, an endo- and exo-β-polyglucosidase, relatively unspecific polyglycosidases, both of which can degrade materials detached from endosperm cell-walls such as barley gums, though only the endo-enzyme can attack the cell-wall itself. The chief function of the exo-enzyme is said to be the removal of the first products of endo-action, in this way preventing inhibition by the high-molecular polysaccharides /

polysaccharides of further action by the endo-β-polyglucosidase. However, an apparent anomaly is presented in a later preliminary report by Anderson (1955) whence it appears that, with green-malt enzymes acting for short periods, viscosity-decreasing activity is directly proportional to reducing group production, suggesting the absence of any exo-activity in the green-malt preparations; these preparations, in fact, resembled in their behaviour bacterial enzyme preparations known to be devoid of exo-β-glucosanase activity. Nevertheless, there is evidence contained in the present work which indicates that there is probably no anomaly, and the behaviour reported by Anderson is what would be expected, given the ratio of endo- to exo-activity actually found in some green malt.

There have been many attempts to assess the malting quality of barley from measurements of the endo-β-glucosanase activity. In the Report of the E.B.C. Barley Committee (1953), however, Thunaeus and Sandegren have pointed out that the "cellulase" or endo-β-glucosanase activity of a barley is influenced by the stage of maturation of the sample. Therefore, it seems that ungerminated barley might be too variable as a test material.

The findings of Thunaeus and Sandegren were verified by Preece, Aitken and Potter (1954), who carried out a survey based on thirty barleys and their malts, while the behaviour of the grain on the floor during commercial malting was also reported. The results obtained gave no support for the view that measurement of endo-β-glucosanase activity might give an indication of malting quality. Some barleys of low activity malted well and some of high activity behaved badly during malting. Potentialities for endo-β-glucosanase activity in barley appeared to be governed varietally, though /

though environment was at least as powerful a factor in deciding the ultimate activity developed in the raw grain, with some varieties perhaps more susceptible to environment than others.

Van Roey and Hupé (1954) also expressed some doubt as to the value of measuring barley endo-β-glucosanase activity as a means of assessing malting quality. These workers reported that a Czech barley, with otherwise satisfactory malt properties, had a lower "cellulase" activity (substrate was ethylhydroxyethyl-cellulose) than a Polish barley with inferior malt properties. "Cellulase" activity of the Czech sample was consistently lower than that of the Polish variety during the germination period while the green malt obtained from the Czech barley was only 20% as active as the green malt of the Polish barley. On the basis of these results and of barley "cellulase" determinations on several European two-rowed varieties, they concluded that "cellulase" activity is completely unrelated to malt quality, thus contradicting the results of the Winnipeg Laboratory. However, van Roey and Hupé (1955) subsequently provided a possible explanation of the anomalous behaviour of their Czech barley. They showed that the Czech variety, with low "cellulase" activity, yielded less barley gum than the Polish barley with high activity. In conclusion, therefore, they said that low gum content could compensate for low "cellulase" activity, a possibility suggested by Preece, Aitken and Potter (loc. cit.).

Bass and Meredith (1955, 1956) decided that the maturation and environmental effects were less likely to affect the endo-activity of green malt, and thus such samples may be more suitable. Despite a loose relationship between the malting quality and endo-activity for the green malts of nine different varieties, the results /

results were still not without anomaly, although the exception could be explained in the same way as the anomaly encountered by van Roey and Hupé.

There has been very little research into the pentosanase system, but these enzymes are of very low activity and so make investigation very difficult; in general, they lie outside the scope of the present report.

EXPERIMENTAL.

Materials examined.

Two samples of barley, an Ymer and a Proctor, were followed through the malting process. The Ymer barley, steeped for three days at 12°C., with daily changes of water, was on the floor for seven days; 26 hr. on the kiln at temperatures rising to 66°C. brought the grain to the hand-dry stage, whereafter it was cured for 33 hr. with a final temperature of 82°C. The Proctor barley, on the other hand, was in the steep for 89 hr., though the conditions applied during the last day of this period were more akin to a normal first day on the floor and, therefore, are regarded as such; the flooring period proper was seven days, during which a maximum temperature of 20°C. was attained by the fifth day. Hand-dry malt was obtained in 22 hr. on the kiln, the temperature having been raised to 66°C., and a temperature of 88°C. was maintained in the 20-hr. curing period. Details of sampling will be apparent from Table I.

Extraction of samples.

Extracts were prepared by the method adopted by Preece and Aitken (1953a) for dry samples, whereas maceration was employed for the wet samples. Strict comparison among the samples examined from /

from day to day was attained by taking 1,000 corns in every instance, and it is important to note that macerations for the preparation of extracts were begun within 1 hr. of removing the samples from the steep or floor. The wet samples were macerated for 5 min. with 200 ml. of 0.6% (w/v) sodium chloride solution followed by continuous stirring at room temperature for 55 min. Continuous stirring for 1 hr. was employed with the raw barley and kiln samples which had been ground in a mill. Therefore, in terms of dry weight, extract concentrations in both series were decreasing from the initial values of approximately 20% (w/v) and 16% (w/v) for Ymer and Proctor barleys respectively to final values in the region of 15% and 13% respectively for the corresponding malts. The resultant extracts were then filtered bright through a fluted filter paper.

Extract activity.

Endo-β-glucosanase activities of the extracts were determined by the method of Preece and Aitken (loc. cit.). The substrate used was a 0.5% (w/v) solution of β-glucosan, prepared from barley according to the method of Preece and Mackenzie (1952). For each determination, 8 ml. of β-glucosan stock solution was mixed with 1.0 ml. of acetate buffer solution (pH 5.0) prepared as described by Palmer (1951) and 2.0 ml. of grain extract; since the stock solution (prepared fresh daily) contained 0.6875 gm. of β-glucosan per 100 ml., it follows that the final 11.0 ml. of reaction mixture contained 0.055 gm. of gum, i.e. was a 0.5% solution. All solutions were adjusted to 25°C. before mixing and the endo-β-glucosanase activities were determined by the viscometric method of Sandegren and Enebo (1952), in which the activities are obtained by observing the decrease in viscosity of the reaction mixture at

approximately /

approximately 10-min. intervals for not more than 90 min. During the later stages of flooring, samples of exceptionally high activity were encountered in which cases it was necessary to employ volumes of extract less than 2 ml., the standard volume being maintained by the appropriate addition of 0.6% sodium chloride solution. This procedure is quite legitimate since Preece and Aitken (loc. cit.) have shown that a linear relationship exists between activity and enzyme concentration.

The results of these determinations are shown in Table I and Figure 1. There will be observed a possible slight fall in endo-β-glucosanase during steeping, an enormous increase during growth and a degree of resistance to inactivation on the kiln. A direct assessment of exo-activity is quite impossible, as the reducing powers of the extracts themselves are far greater than the changes to be measured, and correction at this level is inadvisable. Figure 2 shows the results of a similar investigation, but in this case the grain had been germinated in the laboratory under conditions favouring a more rapid rate of growth; the effect of this treatment on the endo-β-glucosanase is ~~non-existent~~ not similar.

Calculation of extract activities.

Representing the time of flow for water in the viscometer by t_w and that for the reaction mixture at any time by t_s, then the specific viscosity (η sp.) of the reaction mixture at that time is given by the expression $\frac{t_s - t_w}{t_w}$; the reciprocal of the specific viscosity (which will be in the calculations which follow) will be represented by the symbol u. The activity of the extract being examined may then be expressed by the Hultin (1946) principle, which was employed also by Sandegren et al. (loc. cit.) in the form:

E /

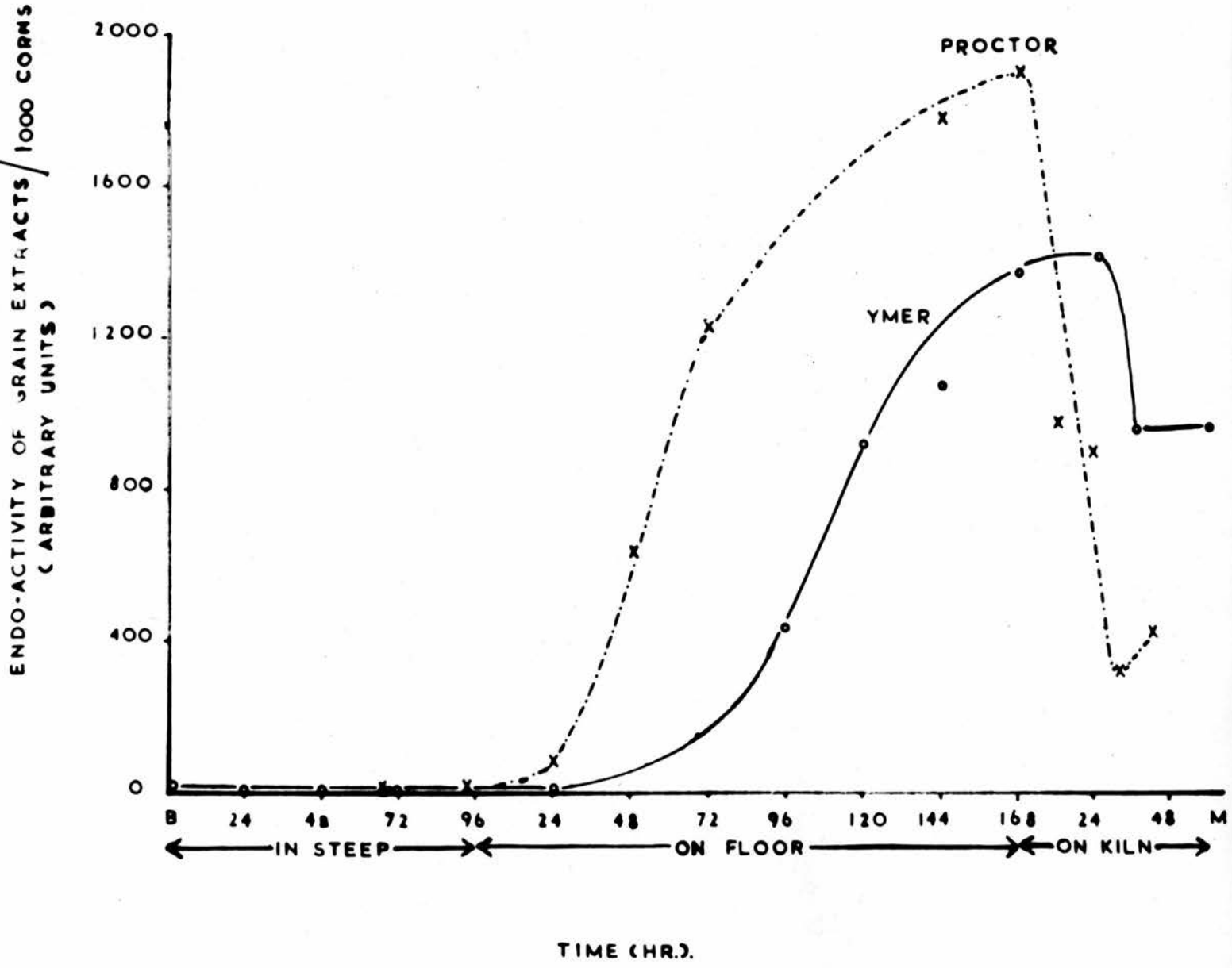


Figure 1.

Changes in the endo- β -glucosanase activity of grain extracts during the commercial malting of Ymer and Proctor barleys.

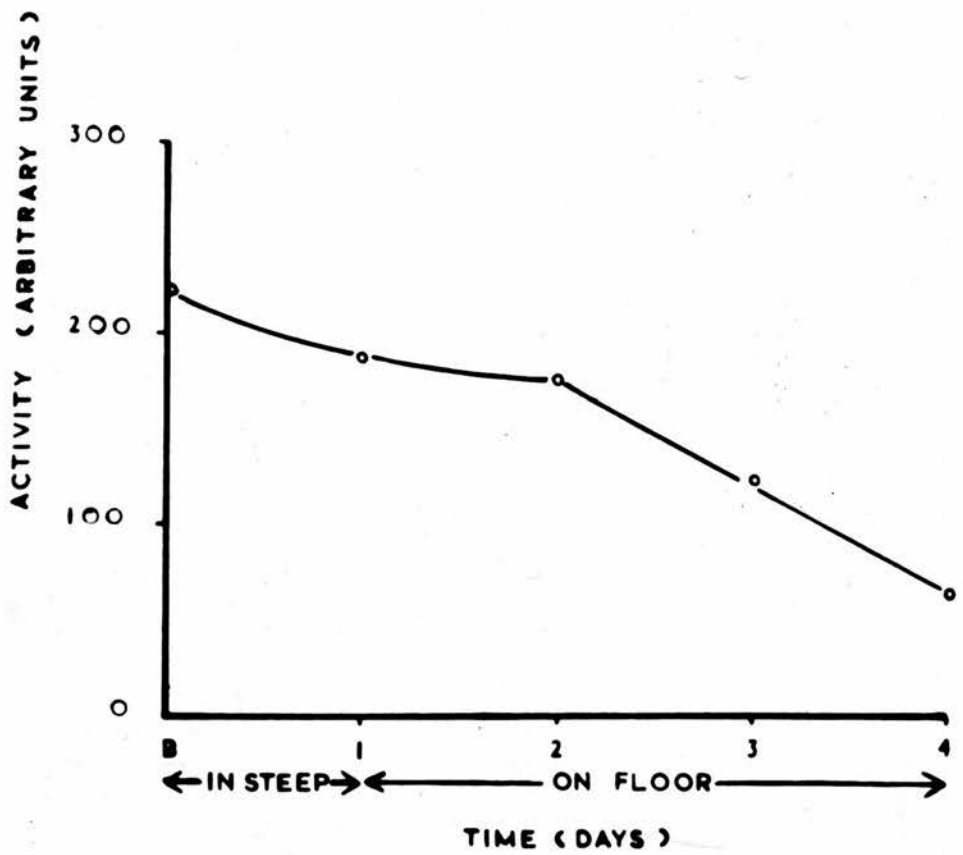


Figure 2.

Changes in the endo- β -glucosanase activity of grain extracts during the laboratory growth of barley (substrate was carboxymethylcellulose).

$$E = k^1.c^2.\frac{du}{dt}$$

where E is the enzyme activity, k^1 is a constant, c the substrate concentration, U the reciprocal of the specific viscosity and t the time in hours from the moment of mixing. The substrate concentration, c, may be considered constant, since its value changes only slightly during the total reaction time of 90 min.; hence the above expression becomes:

$$\frac{E}{k} = \frac{du}{dt}$$

and, since c is a constant, the function $\frac{E}{k}$ may be taken as a measure of the endo-β-glucosanase activity (a) which is readily determinable from the increment of reciprocal specific viscosity with time. This function $\frac{du}{dt}$ is found most readily by plotting a graph of u against t; since the graph is linear over the 90-min. period of examination, its slope $(\frac{du}{dt})$ gives directly the required measure of enzyme activity.

For comparison purposes it was found convenient to express the activity values as units per 1,000 corns. If the function $\frac{du}{dt}$ for x ml. of extract is a_x, then the activity value A per 1,000 corns is given by:

$$A = a_x.\frac{200}{x}$$

since a volume of 200 ml. was employed in each extraction.

Enzyme preparations.

After allowing extracts to autolyse overnight at room temperature, crude unfractionated enzyme preparations were obtained by the addition of four volumes of acetone with stirring, and these preparations, after taking to dryness, were employed for determinations of endo- and exo-glucosanase activities and of cellobiase activity /

activity. It is important to stress the autolysis because it plays a major rôle in the purification of these preparations.

Determination of endo-β-glucosanase activities of the preparations.

For the determination of endo- and exo-β-glucosanase activities the reaction mixtures had the proportionate composition of that employed by Preece and Aitken (loc. cit.), viz., a ratio of 8.0 ml. of 0.6875% (w/v) β-glucosan solution, 1.0 ml. of buffer solution (pH 5.0) and 2.0 ml. of enzyme solution, the concentration of this solution being kept at such a level that decrease of initial specific viscosity of the standard substrate, β-glucosan, to one-half did not occur in less than about 30 min. All conversions, whether for viscometric or reductiometric assessment, were carried out at 25°C. Endo-β-glucosanase activity was determined exactly as described above for determination of the endo-activity of an extract, but results are expressed as increase in reciprocal specific viscosity per hr. per mg. of enzyme preparation.

The values obtained in this way are shown in Table II.

Determination of exo-β-glucosanase activities.

The development of reducing groups was measured on 5-ml. aliquots of reaction mixture withdrawn at convenient intervals up to 4 hr., the Somogyi (1945) copper-reduction method being employed, and the results were corrected for the initial reducing power of the substrate, the value of the glucose equivalent of a particular preparation being smaller the greater the initial viscosity, and for reduction due to the enzyme preparation itself, minor increases in which were allowed for on the basis of concurrently-run controls (autolysis reduced these values to an absolute minimum). However, it was necessary to restrict to 1 hr. those conversions incorporating the more active enzyme preparations (see Table II) because extending /

extending the conversion period to 3 hr. resulted in a negative value for exo-β-glucosanase activity being obtained. It may be that, even with the very low concentration of enzyme employed, the reaction had reached its limit in less than 3 hr., or, alternatively, there is the possibility of transglycosylation. Results, expressed as equivalent mg. of glucose liberated per hr. per mg. of enzyme preparation, are shown in Table II.

Calculation of exo-β-glucosanase activity.

By the very nature of its action, endo-β-glucosanase also liberates reducing groups which will be included in any determination of reducing power. It is necessary, therefore, to correct the values obtained in reducing power determinations for the contribution by endo-β-glucosanase action, and so obtain the total, corrected exo-activity of a preparation.

If it is assumed that β-glucosan is a straight-chain polymer of specific viscosity substantially proportional to the molecular size, at least when this is large, it would appear to follow that rupture of the molecule at a single point remote from the ends would diminish the specific viscosity to one-half, whilst liberating an additional reducing group. The "correction" for the contribution by endo-action is based on this assumption and is calculated as follows:

if x = increase in reciprocal specific viscosity per hr. per mg. enzyme on standard substrate,

y = glucose equivalent (μg.) per mg. of β-glucosan,

z = initial reciprocal specific viscosity,

η_{sp} = specific viscosity,

then time to reach $\frac{\eta_{sp}}{2} = \frac{z}{x}$ hr.

but in a standard conversion (11 ml.) in this time, 55 y μg. reducing /

reducing groups produced

i.e. $\frac{55xy}{z}$ μg. reducing groups per hr.

∴ "Correction" for endo-action is $\frac{55xy}{1000}.\frac{1}{z}$ mg. glucose equivalent per hr.

and for a given preparation of β-glucosan this may be written kx.

The figures for exo-β-glucosanase activity shown in the tables have been corrected for the endo-β-glucosanase contribution to total reducing power.

It follows, therefore, that only figures for reducing group production in excess of this correction value can be taken as evidence of exo-activity. Although the assumptions made above may not apply quite rigidly, there seems to be no reason to suppose that important deviations from the general principle should occur, and the "corrected" figures may be taken, therefore, as giving a reasonable measure of exo-action. It must be borne in mind, however, that this is not necessarily the action of a single enzyme; since, as will be shown in a later section, there is intermediate production of one or more disaccharides susceptible to β-glycobiosidase action, these difference figures represent the joint action of exo-β-glucosanase proper and β-glycobiosidase(s) (the specificity of the β-glycobiosidases is not known and there may be a cellobiase and a laminaribiase present).

Determination of cellobiase activity.

A 0.3437% stock solution of cellobiose was employed and the procedure involved mixing 8.0 ml. of this solution with 1.0 ml. of acetate buffer solution (pH 5.0) prepared according to Palmer (loc. cit.) and brought to temperature equilibrium in a constant temperature bath (25±0.03°C.). The reaction was started by adding 2.0 ml. of enzyme solution which had also been maintained at this temperature /

temperature, the time of mixing noted and the reaction run for the required time. Cellobiase activity was determined on 0.5 ml. of the reaction mixture which was estimated reductiometrically by the Somogyi method (loc. cit.). Substrate and enzyme controls were run concurrently, allowance for their reducing power values being made in the calculation of cellobiase activity which is expressed in terms of mg. cellobiose hydrolysed per hr. per mg. enzyme preparation (see Table II).

As with the determination of the exo-activity of these crude preparations, it was necessary to measure the reducing power after 1 hr. in those conversions incorporating the more active preparations, since extension of this conversion period resulted in lesser or, in some instances, negative values for cellobiase activity.

Calculation of cellobiase activity.

Let c be the number of mg. cellobiose originally present per 0.5 ml. of the reaction mixture,

and let x be the number of mg. cellobiose remaining after t hr. of reaction,

then, $\left[(c-x).\frac{360}{342} \right]$ mg. of glucose are produced.

Let T be the number of ml. 0.005N. $Na_2S_2O_3$ solution equivalent to 0.5 ml. of reaction mixture, after correction for the enzyme control,

$$\therefore \quad T = \left[\frac{x}{0.187} + (c-x).\frac{360}{342}.\frac{1}{0.135} \right] \text{ ml.},$$

where 1.0 ml. of 0.005N. $Na_2S_2O_3$ solution is equivalent to 0.187 mg. pure cellobiose or 0.135 mg. pure glucose.

$$\therefore \quad T = \left[5.34x + (c-x)7.79 \right] \text{ ml.}$$

$$= \left[7.79c - 2.45x \right] \text{ ml.}$$

$$\therefore \quad x = \frac{7.79c - T}{2.45} \text{ mg. per 0.5 ml. reaction mixture.}$$

$\therefore$ /

$\therefore \left[c - \frac{(7.79c - T)}{2.45} \right]$ = number of mg. cellobiose hydrolysed per 0.5 ml. reaction mixture after $\underline{t}$ hr.

. . In the standard conversion (11 ml.) the cellobiase activity is given by

$$\left[c - \frac{(7.79c - T)}{2.45} \right] \times \frac{22}{t.w}$$ mg. cellobiose hydrolysed per hr. per mg. enzyme

where w = weight of enzyme employed in standard conversion.

The results for the three aspects of activity are gathered together in Table II with the yields of enzyme mixture. The pattern is the same with both barley samples. Yields decrease during steeping, increase during flooring and decrease again on kiln; bearing in mind that these are crude preparations, it will be realised that these changes represent, very largely, variations in amounts of contaminating matter and, as such, follow broadly the pattern that would be expected. It will be shown in a later section that the trend of activity is different where purified preparations are employed. From Figure 3 it is seen that endo-activity shows little change in preparations from samples from the steep and from the earliest stages on the floor; after 96-120 hr. on the floor there is a very great increase in activity, reaching a maximum during the early stages on kilning and thereafter falling off somewhat. The great increase was observed only for Ymer preparations, whilst the Proctor series, although following the same general pattern, never approached a similar high level of activity. Exo-activity varies rather more irregularly (see Figure 4), but a maximum is attained in preparations from samples taken towards the middle or end of flooring, the fall in activity being such that this activity cannot be detected in the finished malt preparations. Figure 5 shows the confusing nature of the cello-biase preparations, but it will be noted that there is no real

parallelism /

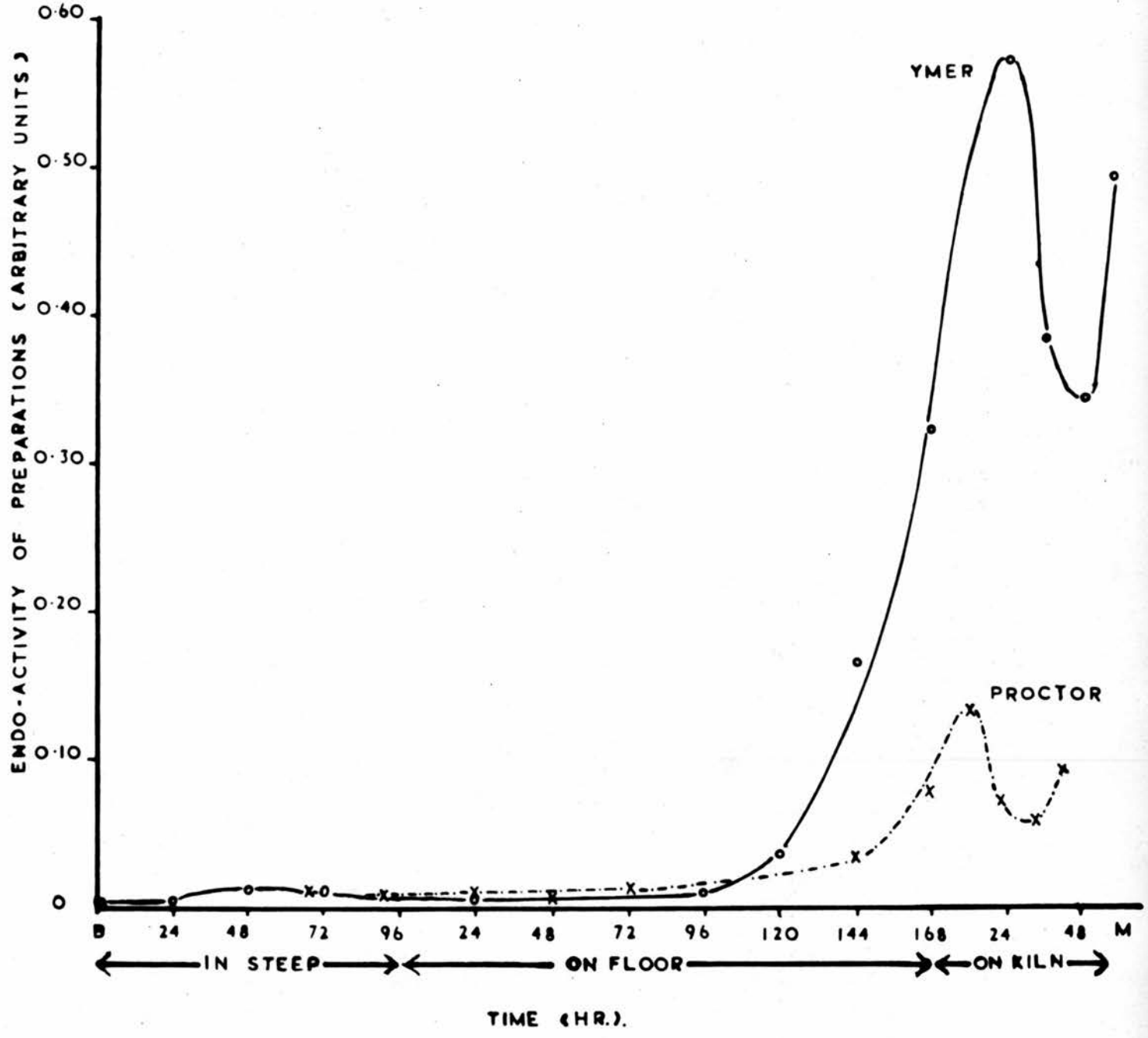


Figure 3.

Changes in endo- β -glucosanase activity of enzyme preparations obtained from grain during the commercial malting of Ymer and Proctor barleys.

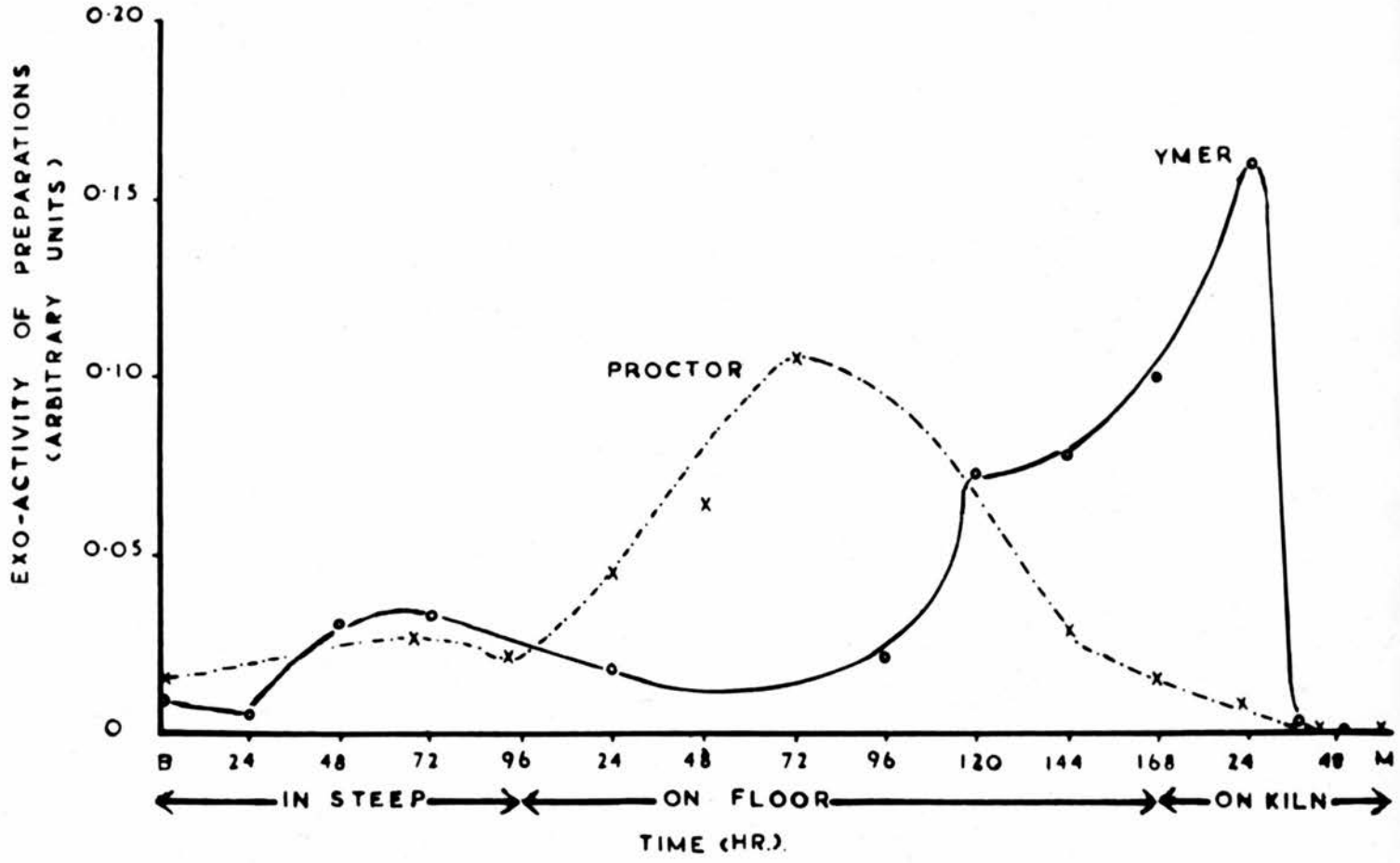


Figure 4.

Changes in exo- β -glugosanase activity of enzyme preparations obtained from grain during the commercial malting of Ymer and Proctor barleys.

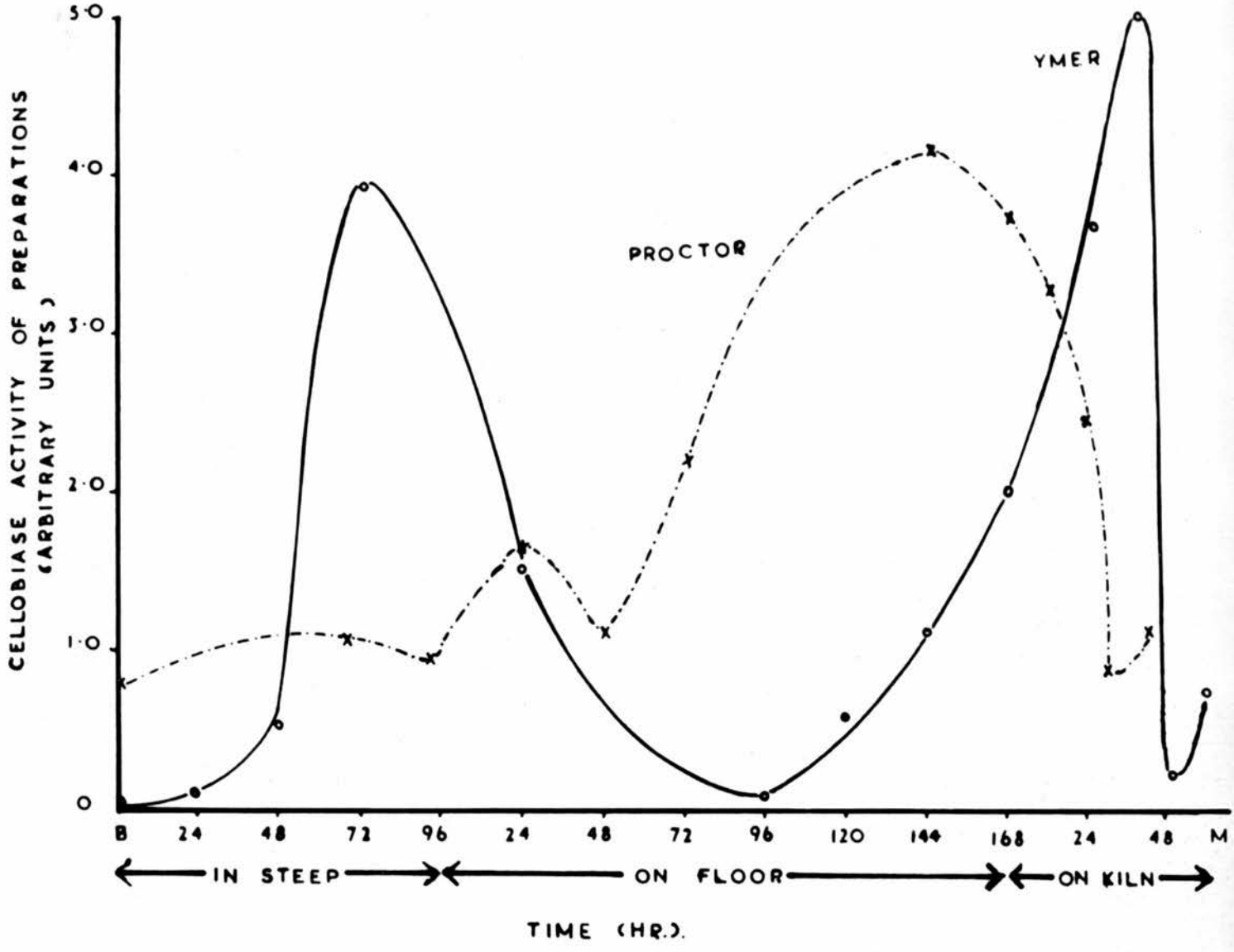


Figure 5.

Changes in cellobiase activity of enzyme preparations obtained from grain during the commercial malting of Ymer and Proctor barleys.

parallelism between the three aspects of activity, emphasising their independent nature.

Effect of mashing on endo-β-glucosanase activity of malt.

The malts of the Ymer and Proctor barleys already discussed were subjected to mashing treatment by the standard Institute of Brewing technique. 50 gm. of crushed malt were stirred into 350 ml. of distilled water at a temperature of 68-69°C., after which the temperature of the mash was maintained at 65.5°C. for 75 min. During this period the mash was stirred at 10-min. intervals. Endo-β-glucosanase activities were determined in the standard conversion (11 ml.) by the viscometric technique, 2-ml. aliquots of extract being removed from the mash every 10 min. up to 75 min., the first of which was taken 5 min. after the start. In order to exclude small particles of grain from these aliquots it was necessary to withdraw them through several layers of muslin. From Figure 6 is seen the manner in which increasing time of extraction at 65.5°C. affects the level of endo-activity, the initially small activities being eliminated after 65-70 min.

The extract concentrations in these experiments were approximately 14% (w/v), which compares favourably with the extract concentrations employed in the investigation of the behaviour of endo-β-glucosanase activity during malting. Figure 6 shows that, after 5 min., the endo-activities of the extracts are 0.26 and 0.15 units of activity for Ymer and Proctor malts respectively. In comparison, from Table I it is seen that the extract activities for Ymer raw barley and malt are 16.0 and 970.0 units respectively, while the activities of the extracts of Proctor raw barley and malt are 10 and 428 units respectively. Therefore, compared with the activities of those extracts obtained at room temperature, the endo-β-glucosanase /

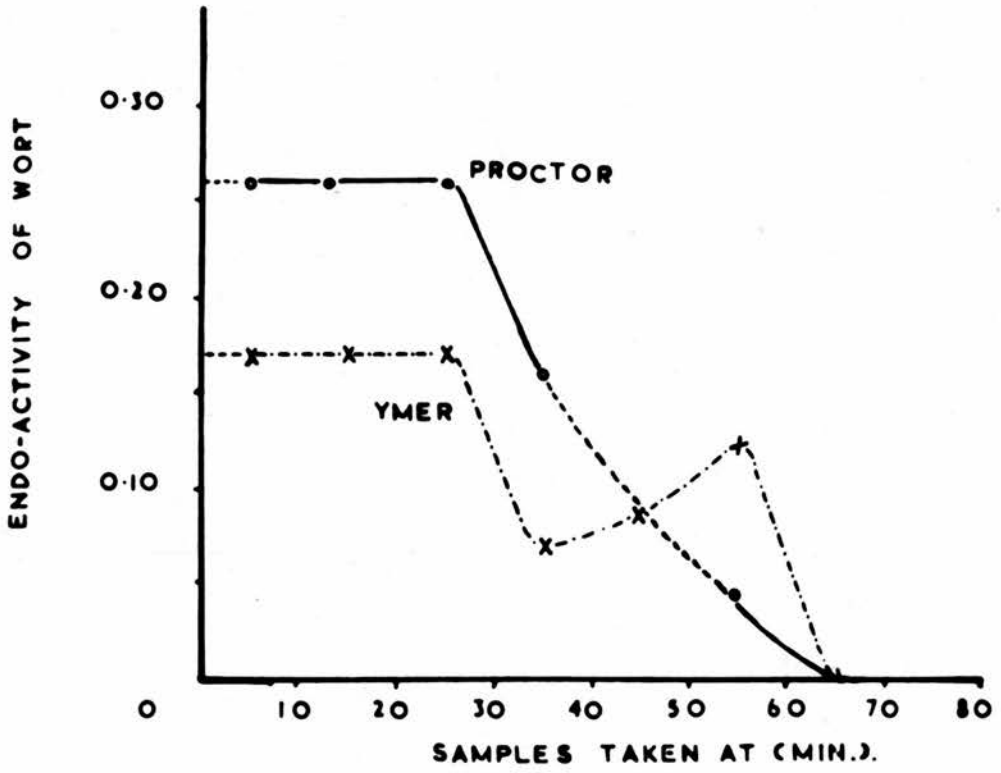


Figure 6.

The endo- β -glucosanase activity of wort samples taken at intervals during the extraction of Ymer and Proctor finished malts with water at 65.5°C.

TABLE I.

Endo-β-Glucosanase Activity of Grain Extracts during Malting.
(Results are based on 1,000-corn samples)

	Ymer barley		Proctor barley	
Stage	Time (hr.)	Endo-β-glucosanase	Time (hr.)	Endo-β-glucosanase
Raw barley	-	16 *	-	10 *
Steep 1	24	10	-	- †
2	48	9	65	7
3	72	8	89	16
Floor 1	24	13	24	83
2	96	440	48	636
3	120	920	72	1232
4	144	1080	144	1792
5	168	1380	168	1896
Kiln 1	26	1416	12	984
2	38	966	22	904
3	50	966	32	320
4	59	970 ≠	42	428 ≠

* The activities of the raw barleys on the scale of Preece and Aitken (1953) (i.e. per 100 gm.) were: Ymer 38; Proctor 29.

† Not examined.

≠ Finished malt.

TABLE II.

β-Glucosanase and Cellobiase Activities of Enzyme Preparations from Grain at Successive Stages during Malting.

Stage	Ymer barley				Proctor barley			
	* Yield	Arbitrary units based on standard 11 ml. substrate + Endo-β-glucosanase	Exo-β-glucosanase	Cellobiase	* Yield	Arbitrary units based on standard 11 ml. substrate + Endo-β-glucosanase	Exo-β-glucosanase	Cellobiase
Raw barley	1300	0.004	0.009	0.03	715	0.007	0.015	0.80
Steep 1	720	0.007	0.005	0.11	-	-	-	-
2	475	0.014	0.031	0.55	360	0.011	0.027	1.07
3	485	0.010	0.034	3.95	416	0.009	0.021	0.94
Floor 1	525	0.007	0.018	1.52	504	0.010	0.045	1.66
2	1058	0.011	0.021	0.10	760	0.006	0.064	1.12
3	1027	0.038	0.074	0.60	1190	0.013	0.105	2.21
4	1207	0.167	0.079	1.13	1400	0.035	0.029	4.19
5	1248	0.323	0.100	2.03	1427	0.077	0.015	3.78
Kiln 1	1152	0.571	0.161	3.71	1295	0.133	0.000	3.30
2	1072	0.383	0.004	5.04	1331	0.074	0.009	2.47
3	1153	0.343	0.000	0.25	1256	0.060	0.005	1.87
4	862	0.496	0.000	0.78	1052	0.092	0.000	2.16

* Mg. per 1,000 corns; raw barley and kilned samples were ground, those from the steep and floor being macerated.

+ As in Table I.

TABLE III.

The Variation of Endo-β-Glucosanase Activity with Kilning Temperature.

Kilning temperature (°C.)	Percentage loss of endo-activity
82	33
85	50
88	77

β-glucosanase activities of the worts from both samples of malt are almost negligible. Indeed, employing an extract of 0.26 units of activity, a conversion time of approximately 42 hr. would be necessary to reduce the specific viscosity of the substrate (13.40) to one-half.

DISCUSSION.

The way in which malting of barley influences the endo-β-glucosanase activity associated with material soluble in 0.1 M sodium chloride solution is shown in Figure I. After a slight decrease in activity during steeping, there is an enormous increase during flooring to an ultimate maximum at the end of flooring or in the early stages of kilning at a value 90 times (Ymer) or 190 times (Proctor) what it was originally; during kilning, there is some degree of loss of activity according to the conditions imposed, the Ymer and Proctor malts examined being respectively 60 and 42 times as active as the original barleys. The Proctor malt has been affected to a much greater extent, probably the result of the higher curing temperature (88°C.) employed. Sandegren and Enebo (1952), working on a similar problem, have shown that kilning at 85°C. effects a loss of 50% of the original activity. Therefore, it appears that the higher the kilning temperature the greater is the loss of endo-β-glucosanase activity (Table III). However, Sandegren et al. employed soluble derivatives of cellulose as substrates in their determinations, hence the results, in the light of recent evidence by Thomas (1956), are not directly comparable with the present observations.

In the past, many workers have employed the results of endo-β-glucosanase determinations to predict the malting potentialities of a barley, but as a consequence of the enormous possibilities of increase /

increase in activity during malting it may be suggested that the initial activity is of little significance, and this may go a long way towards explaining why determination in the barley itself has proved of so little value. This point is illustrated by the results contained in Table I in which it is observed that Proctor barley extract is less active towards β-glucosan than an extract of Ymer barley, but the Proctor variety is greatly superior as a malting barley. An explanation of this anomaly may be afforded from the investigation of Thunaeus and Sandegren (1953) to which reference has already been made. As a test material, therefore, ungerminated barley is too variable, and later the work of Bass and Meredith (1955) with green-malt extracts also gave anomalous results. It may well be that the increase during malting is, in any case, so great that excess endo-β-glucosanase activity is present in the majority of instances and that some other factor than this enzyme controls essential cell-wall polysaccharide changes, β-glucosan itself being readily disposed of. Admittedly, maturation and environmental effects are less likely to affect the endo-activity of green malt, but it may be necessary then to take into consideration the initial treatment of the grain prior to flooring. Again, taking the present results (Figure 1) as an example, it is observed that Proctor barley, with an initially lower endo-β-glucosanase activity than the Ymer sample, attains a much greater maximum. It is suggested that this may be a direct result of the treatment given in the steep, at which stage the Proctor barley was subjected to an additional 24-hr. period of "dry steeping", and therefore it is highly probable that this treatment has had the effect of increasing the level of endo-activity to the maximum attained at the hand-dry stage. Preece

and /

and Mackenzie (1958[36]) suggested that, to overcome these low initial enzyme activities, the grain may require very careful nursing in the early stages, perhaps by way of extra steeping and of special attention in the early days on the floor. Thus, it is realised that a low initial endo-β-glucosanase activity can be overcome by suitable treatment and, furthermore, the present results lend support to a recent suggestion put forward by van Roey and Hupé (1955) to explain the anomaly presented by the Czech and Polish barleys mentioned earlier. They concluded that low gum content could compensate for low endo-β-glucosanase activity, and it will be observed later (Section 2) that Proctor barley has only 54.5% of the water-soluble gums extractable from Ymer barley under the same conditions.

The endo-activities of crude enzyme preparations (Figure 3) at the various stages of malting are of interest, the most active material being obtained at the early stage of kilning (Ymer 130 times; Proctor 20 times as active as the original barley preparations). Although the pattern is similar in both series, the activities of the Proctor preparations do not approach the high level of activity attained in the Ymer series. However, this result may not be too surprising because it is known that other aspects of the special metabolic system in the barley grain increase in parallel with endo-activity, e.g. amylolysis (Figure 7), and Massart (1949) has shown that proteolysis increases greatly after 3-5 days on the floor. Therefore, it is possible that, having accelerated the rate of increase of endo-β-glucosanase activity, the initial treatment of the Proctor barley has also had the effect of increasing the autolysis of active material. It is perhaps significant that the laboratory growth of barley, during which /

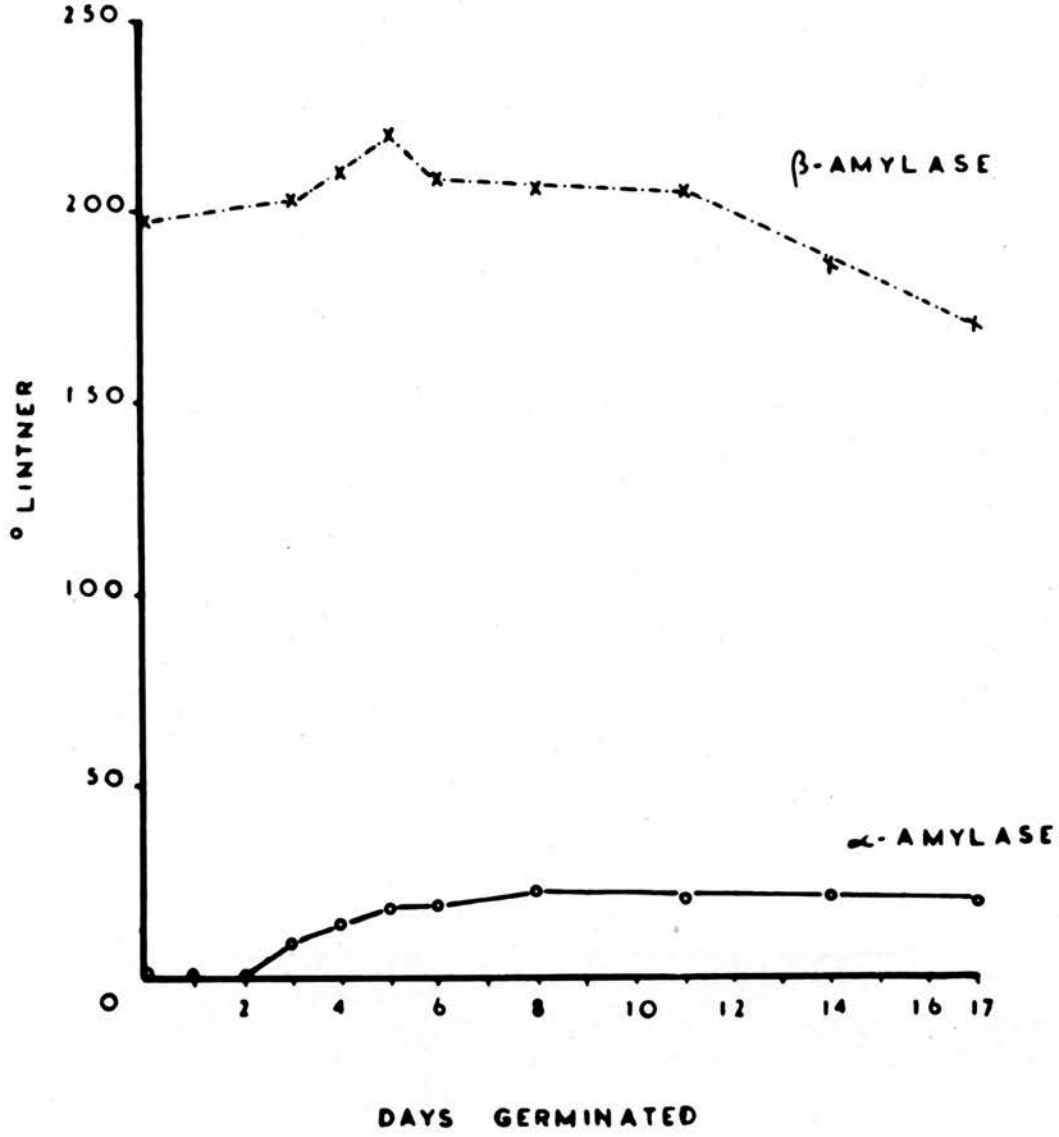


Figure 7.

Changes in α- and β-amylase activities during the growth of barley.

which excess water was present, gave no increase in endo-β-glucosanase activity (Figure 2), presumably the result of extensive autolysis under extreme conditions. On the other hand, it must not be assumed that the endo-activity developed during the growth of Proctor barley, in vivo, is of a similar low order, because there is evidence (Section 2) that whilst Ymer and Proctor barleys possess approximately equal quantities of initially-water-insoluble hemicelluloses, in the finished malts approximately 100% more is obtained from ~~Proctor~~ Ymer. Therefore, it appears that the breakdown of initially insoluble hemicellulosic material has been more complete in the malting of Proctor barley and so the results shown in Table I, determined shortly after extraction of the grain, present a more accurate picture of the behaviour of the endo-β-glucosanase system during modification. Thus, it is clear that the results of in vitro experiments must be interpreted with the utmost caution.

Considered in terms of recovery (yield × activity of preparations compared with activity of extracts as in Table I) it will be observed from Figure 8 that, whilst recovery from raw barley and samples from the steep may be of the satisfactory order of 40-70%, and whilst the figures for Ymer and Proctor finished malts are, respectively, about 50 and 20%, the recovery of activity from samples taken in the middle stages of flooring is extraordinarily low, of the order of 2-4%. Again, it seems possible that this may be explained by autolysis of enzyme material during the stages of preparation; if this is so, the figures for endo-activities of extracts may themselves, to a lesser extent, under-estimate the true potentialities of the grain. Furthermore, Enebo et al. (1953), investigating the effect of added sugars on the activities of crude green-malt /

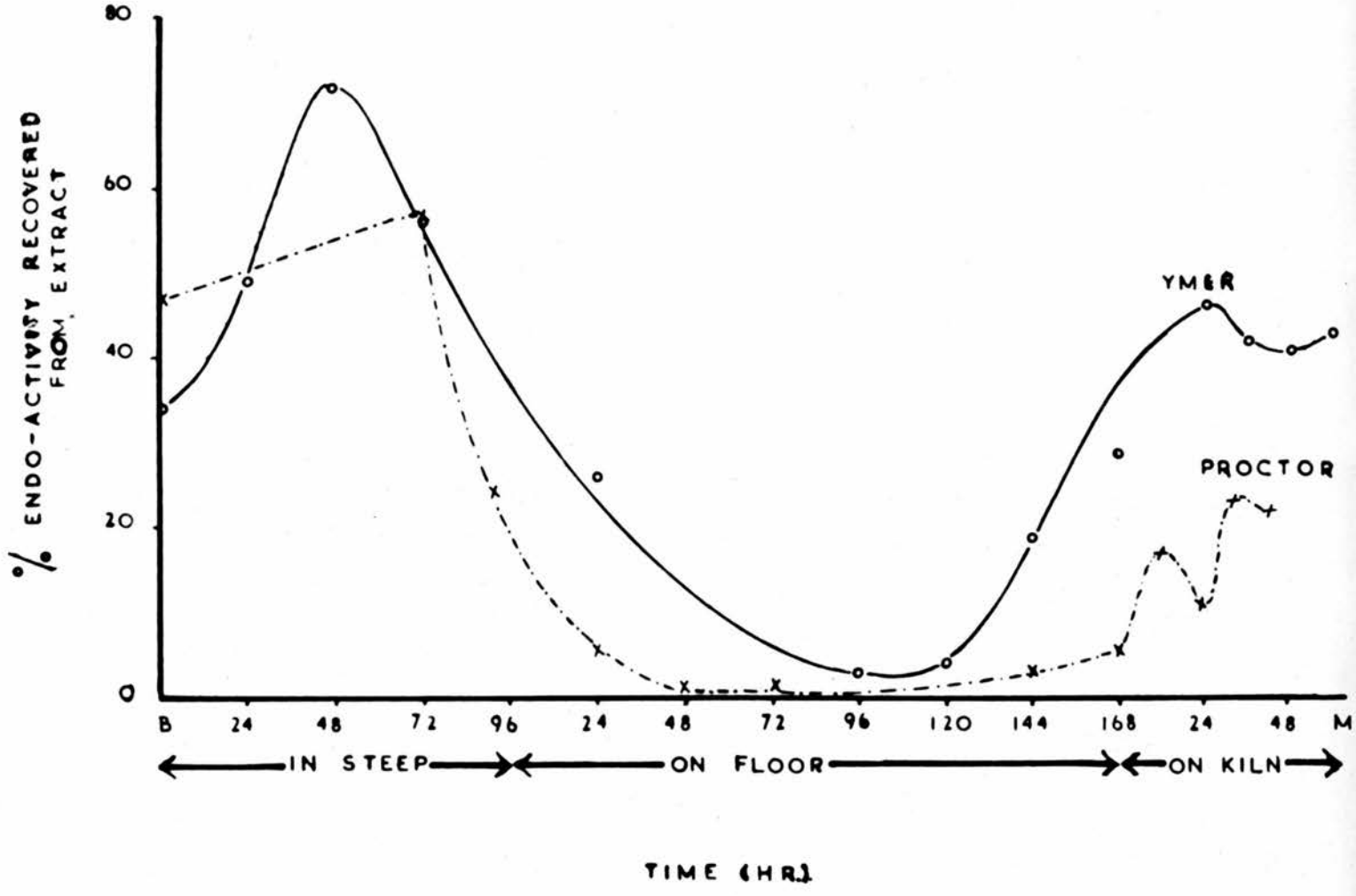


Figure 8.

Changes in the percentage recovery of endo- β -glucosanase activity from grain extracts during the commercial malting of Ymer and Proctor barleys.

green-malt extracts and purified green-malt preparations, found that the addition of glucose or xylose activated the purified preparations, but had no effect on the activities of crude extracts. They explained this by assuming that the extracts already contained sufficient amounts of these monosaccharides, further additions having no effect. It may be, therefore, that the observed low recoveries of endo-β-glucosanase activity from the extracts are, at least in part, the result of the removal of these sugars on precipitation of the enzymes. The subsequent increase in recovery could be explained by the liberation of further amounts of active material or by the further degradation of concomitants. It is interesting to note that the point at which recovery is minimal coincides with the stage at which the greatest changes seem to be taking place in the grain. The degree to which the concomitants present in these enzyme preparations, obtained by acetone precipitation, influence the activity patterns is not known, but it will be shown later (Section 3) that all evidence points to the concomitants being largely carbohydrate in nature. The increased activities of finished malt preparations in comparison with those of green malt may, in part, be ascribed to the continued degradation of the accompanying materials during kilning to a degree of polymerisation at which they are scarcely precipitable.

It is unfortunate that no means exists as yet for determining the recovery of exo-β-glucosanase activity, but if losses of this are no greater than of endo-activity, the exo-activity finds its maximum at the middle or end of flooring at no more than 18-19 times the initial values (Figure 4). However, it is surprising, in view of the great differences between the maxima of the endo-β-glucosanase activities of the preparations, that there is virtually /

virtually no difference in the maximum activities attained by the exo-enzymes. From Figure 4 it is observed that exo-β-glucosanase appears to be susceptible to the high temperatures attained in the kiln, although there are indications that a fall in activity occurs before the start of kilning.

It is significant that, when determining exo-β-glucosanase activity of these preparations with high endo-activities, a 3-hr. conversion resulted in a failure to detect exo-activity, whilst repetition of the determinations after 1 hr. and with lesser concentrations of enzyme preparations revealed, in some cases, a small but measurable activity. It is thought that this was the result of the reactions attaining their limits in less than 3 hr. with the enzyme concentrations employed; thus, with the level of activity now known to be present, no activity would be detected. The possibility of transglycosylation cannot be ruled out in such cases since the polysaccharide chain is broken down very rapidly into small molecules and so the possibility of transfer reactions involving glucosyl residues cannot be overlooked.

As the ratio of exo- to endo-activity falls, it is clear that the proportionality between reciprocal specific viscosity and reducing group production characteristic of endo-activity will be the more closely approached. This may be the explanation of an anomaly reported by Anderson (1955), who stated that, with green-malt preparations acting for short periods, viscosity-decreasing activity appeared to be directly proportional to reducing group production, suggesting the absence of any exo-activity.

A consideration on the following lines of the isolated β-glucosanase systems of raw barley, green-malt and finished-malt preparations is interesting. The ratio of exo- to endo-activity in /

in an Ymer raw barley preparation is approximately 2 : 1, of Ymer green malt about 1 : 3 and in Ymer finished malt there is no detectable exo-activity; therefore, from a degradative viewpoint these are distinct systems. For instance, the green-malt system now resembles, in gum-splitting propensities, the β-glucosanase systems of oats (1 : 5) and maize (1 : 16), and it is expected that the oligosaccharide patterns produced by the action of raw barley, green-malt and finished-malt enzymes on β-glucosan will show similar resemblances.

Little information is available regarding the solubilising action of the β-glucosanase system, but recently Preece and MacDougall (1956), having shown that the pentosanase activity of green malt increased to approximately 12 times the original activity of the raw barley (enzyme extracts and preparations obtained from the Proctor samples employed in the present work), extended their studies to include an investigation of the solubilising action of green-malt (8-day) extracts and preparations upon barley grains from which starch and initially-water-soluble hemicelluloses had been removed. Material consisting of 6% and 30% glucosan was obtained in the reactions involving extract and preparation respectively and therefore it appears unlikely that the solubilising action is entirely mechanical (a concurrently-run control gave no product whatsoever). Furthermore, chromatographic examination of the reaction liquors revealed a series of glucose oligosaccharides, pentoses being absent. However, after 24 hr. and employing an enzyme preparation from a 12-day malt, arabinose, xylose and pentose oligosaccharides were detected, with but a trace of glucose. These data agree remarkably well with the conclusions reached by Preece and Mackenzie (1953) from the results /

results of autolytic experiments. They stated that the composition of the product obtained will depend on the balance of enzyme activities involved, and they observed that, with β-glucosan, the degradative effect quickly surpasses that of hemicellulose solubilisation, whilst with pentosan substrates this is not the case, the autolytic enzymes over a protracted period of time being less effective than those causing hemicellulose solubilisation. That the degradation of β-glucosan is much quicker than pentosan breakdown receives further support in that the product obtained after a reaction time of 4 hr. contains only 6% glucosan and, as we have observed, this degradation is very extensive over a short period of time. The product obtained by the action of the enzyme preparation has a composition somewhat similar to the composition of barley hemicelluloses (Section 2) obtained by alkaline extraction, and therefore an initially insoluble reserve of these materials is suggested.

In Figure 7 is shown the effect of growth upon the α-glucosanase system of barley. There is a relatively low α-amylase activity up to the ~~fourth~~ second day of growth, after which there is a sharp increase in activity, whilst β-amylase shows only a small increase during growth. These results suggest a similarity between the α- and β-glucosanase systems. Endo-β-glucosanase and α-amylase, however, have a few points in common, namely, they are both present in the original grain in trace amounts, they produce oligosaccharides from the respective substrates on prolonged action, both are relatively stable when aqueous solutions are heated to 70°C. for 15 min., but when an extract of malt is heated to 65.5°C. they are destroyed rather quickly and each shows a degree of resistance to heat on kilning. Considering the

exo- /

exo-β-glucosanase and β-amylase enzymes, there is a moderate amount of each in raw barley, they produce disaccharides from their respective substrates, both are susceptible to -SH inhibitors and they are destroyed to a greater or less extent when green malt is heated at 82-88°C. However, the similarity between the two systems is not without anomaly because α-amylase is destroyed in 1 hr. in acid (pH 3.3) whereas endo-β-glucosanase is almost unaffected. The similarity can be extended to include maltase with the α-glucosanase system and cellobiase with the β-glucosanase system, both enzymes acting by hydrolysing their respective disaccharides. It would seem, therefore, that the α- and β-glucosanase systems do have a similar mode of attack, a similar behaviour on germination, and an almost identical response to physical conditions.

In view of the negligible level of endo-β-glucosanase activity in wort (Figure 6), even soon after the start of extraction at 65.5°C., it seems certain that very little further degradation and solubilisation of initially soluble and initially insoluble hemicelluloses respectively can take place at this temperature even taking into consideration the fact that the enzymes in wort may possess stability characters rather different from those which they show in water. The pattern observed in the figure is governed by two factors, firstly, the rate of extraction of the active material and, secondly, the temperature effect, the low initial activity of the wort being eliminated finally after 65-70 min. Under these conditions of extraction, therefore, the contribution of hemicellulosic materials must be almost entirely from these already in a soluble form in the raw materials present. Support for these results is obtained from the investigations of

Sandegren /

Sandegren and Enebo (loc. cit.) in which they observed the elimination of endo-β-glucosanase activity ("cellulase") at 66°C., but they do not state the period which elapsed before this occurred. In an effort to estimate the extent of degradation of β-glucosan, if any, it is estimated that with the initial activity, 0.26 units calculated according to Preece and Aitken (1953), of Proctor malt extract, a conversion time of approximately 42 hr. would be necessary to reduce the specific viscosity of β-glucosan (initial specific viscosity 13.40) to one-half, at 25°C. This period would be considerably extended since the temperature of the extract is 65.5°C. and also because the degree of polymerisation of malt β-glucosan is quite low (in Section 3 it will be shown that, at specific viscosity values less than 2.0, the degradation of β-glucosan, independent of enzyme concentration, occurs very slowly even under standard conditions). Therefore, it seems safe to assume in the light of known facts that the contribution by the pentosanase system will be no greater than that of the β-glucosanase system; hence, solubilisation and degradation of hemicellulosic substrates, under these conditions of extraction, will be negligible at the very most.

SUMMARY.

The independent nature of the three aspects of activity has been shown quite clearly.

There is evidence for enzyme capabilities which

(a) render insoluble hemicelluloses soluble;

(b) degrade the soluble hemicelluloses, at first, apparently by decreasing viscosity, the pattern being similar to that of α-amylase action. There is also evidence which suggests that the degradation of β-glucosan and pentosan (araboxylan) goes as far as the monosaccharide stage, the degradation of the former being very rapid.

A saccharifying enzyme, exo-β-glucosanase, is also present and it resembles β-amylase, whilst a cellobiase has been shown to be present also, thus completing the similarity between the α- and β-glucosanase systems (with maltase) of barley.

It has become apparent also that the manner in which β-glucosan is degraded will depend on the stage of growth of the grain, because raw barley, green malt and finished malt have distinctly different exo- to endo- ratios, the green-malt system resembling that of maize and oats.

The conclusion can also be reached that very little dependence ought to be placed upon measurement of endo-β-glucosanase activity of raw barley or green malt as means of predicting malting quality because so many factors are involved: namely, environment and stage of maturation of the grain, initial treatment by the maltster in the steep and, furthermore, there is always an excessive endo-activity associated with green-malt. However, it is significant that the greatest changes in the activity of enzymes associated with the metabolic system seem to take place around 96-120 hr. on the /

the floor.

Solubilisation and degradation by endo-β-glucosanase during extraction of malt at 65.5°C. must be, at the very most, negligible.

SECTION 2.

The Malting of Barley: Examination of the Hemicellulosic Materials.

INTRODUCTION.

It has been observed that growth of the barley corn results in the dissolution of the cell-walls of the endosperm leaving only a transparent skeleton (Grüss loc. cit. and Ling loc. cit.), thus giving the characteristic friable grain of barley malt. This physical change, known as modification, arises in part from the combined solubilisation and degradation of initially insoluble and initially soluble hemicellulosic materials. In this way it was concluded by Dickson and Shands (1941) that the cell constituents are exposed to subsequent action by amylolytic and proteolytic enzymes.

O'Sullivan (1892), having isolated α-amylan (barley gum B_2, a pentosan-contaminated β-glucosan) from barley, failed to detect it in malt, whilst Piratzky and Wiecha (1938) demonstrated the increased production of this material in the early stages of growth and its virtual elimination thereafter. More recently Meredith et al. (1951) also reported the absence of the laevorotatory fraction from malt. These important early observations of the degradation of β-glucosan were clearly confirmed by Preece and Mackenzie (1952). Their method involved ammonium sulphate fractionation of water-soluble, non-starchy polysaccharides isolated from extracts of enzyme-inactivated barley, barley which had been submitted to the brief action of barley enzymes, and from extracts of the finished malt of the same raw barley. In this way, laevorotatory glucosan was obtained at two different solubility levels from raw barley and from enzyme-treated barley, but only at the higher /

higher level from malt. Moreover, the yield from malt was comparatively insignificant, whereas the enzyme treatment of barley diminished the amount of the less soluble and increased the yield of the more soluble fraction, but with concurrent fall in viscosity of both fractions. This brief treatment of barley with its own enzymes was taken to simulate the behaviour of the β-glucosan during the early stages of growth.

Hall, Harris and MacWilliam (1954, 1955), working on the carbohydrates of malt and wort, have shown that Plumage-Archer and Carlsberg barleys of different seasons produce a greatly increased yield of water-soluble glucosan during malting, a maximum being attained in the green malt prior to kiln-drying. These results for the behaviour of β-glucosan during malting agree remarkably well with those obtained by Preece and Mackenzie (1953), who attributed the decreased recovery of β-glucosan in the later stages to the degradative effect quickly surpassing that of hemicellulose solubilisation.

Preece and Mackenzie (1952) simultaenously isolated pentosan-rich fractions which were precipitated by ammonium sulphate at three higher solubility levels. It is interesting to note that, contrary to β-glucosan behaviour, the yields of pentosan increased from barley to malt. From this they concluded that, despite some sharp viscosity changes at particular levels, the overall contribution of these fractions to extract viscosity may only show a relatively small decline. The non-precipitable material, which has little effect on viscosity, increased sharply during malting. This fraction is largely glucosan of dextrinous type. Therefore, it seems that, since the total high-molecular soluble pentosans increase in amount during malting, the malt gum owes its origin

not /

not only to the initially-water-soluble barley gums but also to the initially insoluble material which may be either of gum-like nature or the nature of typical hemicelluloses. These observations are in accord with those of earlier workers.

Schöne and Tollens (1901) were the first to examine the pentosan content of seeds, e.g. barley, wheat and peas, before and after germination. In all cases they observed a slight increase in pentosans in the germinated seeds and this they attributed to a production of these substances from starch. In the same year, Windisch and Hasse re-examined this problem and concluded that a marked increase takes place, the products accumulating in the malt itself when germination is not unduly prolonged. However, there was no change in the proportion in the endosperm and this they took to indicate that the pentosans of the endosperm do not function as reserve materials but that the additional material is derived from other carbohydrates. Continuation of this work by Windisch and van Waveren (1909) indicated that a considerable loss of pentosans takes place from the husk during steeping.

The work of Baker and Hulton (1917) led to some very significant conclusions. They also observed the increase in furfurogenic substances during growth whilst they demonstrated that barley embryos, excised and grown in the dark on sand moistened with a solution of cane sugar, also gave an increase in furfuraldehyde yield, this accompanying an increase in weight of the embryos themselves. Embryos grown normally with their own endosperm gave an increase which accompanied a similar decrease in the endosperm. It appeared, therefore, that translocation of pentosans was taking place, this being made possible by the action of an enzyme system capable of rendering pentosans soluble and diffusible. The presence of this enzyme /

enzyme system ("cytase" or "hemicellulase") in green malt was demonstrated by these workers. This enzyme system, which solubilised constituents of barley, malt husks and spent grains, also produced free pentose sugars.

Van Laer and Masschelein (1923) made a very complete study of the pentose changes during malting. They reported an approximately 4% loss of total pentosans during steeping, this loss being almost entirely from the husk. However, they observed an increase in pentosan during growth both in the endosperm and in the embryo, whilst kiln-drying was shown to cause a further increase, chiefly in the endosperm. The kilned malt possessed 20% more pentosan than the corresponding barley.

An interesting review of the pentosan problem in malting was provided by Fink and Hartmann (1934). They stated that the change from the hard barley corn to the friable grain of malt is probably largely the result of changes in the cell-walls of the endosperm, and is accompanied by an increase in the soluble pentosans of the corn. Therefore, this seemed to suggest that it might be possible to develop a method for assessing the degree of modification of a malt from the results of furfuraldehyde determinations. The merit of such a determination would be that its results should theoretically be more closely allied to the mechanical modification of the grain as envisaged by the practical maltster than determinations based on changes in nitrogenous substances.

Fink (1935) and Lüers (1935), pursuing the subject still further, found that the total pentosans increased only slowly during germination, but they observed an approximately four-fold increase in soluble pentosans in the kilned malt. The maximum was reached /

reached on the sixth day of an 8-day period of growth, it appearing that, after 5-6 days, the further production of soluble pentosans is balanced by synthetic processes in the developing embryo. Hence, Fink concluded that the determination of soluble pentosans as a measure of modification does not offer much promise, since the measured production ceases before modification is complete.

Enders, Saji and Schneebauer (1938) have expressed a similar doubt as to the regular value of such a method for assessing modification.

Preece (1940) confirmed the increase in furfuraldehyde yield as a result of growth, but he also pointed out that the increase seemed to take place in all parts of the grain during the early stages. He detected the expected increase in pentosans in the embryo which is maintained throughout the process, and also a more surprising, but small, increase in husk pentosans. It is extraordinary that an increase should be recorded in the husk, which is usually regarded as inert tissue, although it may be that this increase is merely a mechanical one, due to diffusion from other parts of the grain. The initial increase in soluble pentosans from the endosperm was not maintained and the subsequent decrease was explained on the basis of either further transformation of the substances concerned or of translocation to the embryo. Preece (loc. cit.) states that enzymic action may be responsible for these increases, but adds that there is the further possibility that part, at least, of the increase may be the result of decarboxylation of polyuronide material. This latter assumption is in agreement with results published earlier by Lüers and Collignon (1939).

Preece, Ashworth and Hunter (1950) summarised all previous work /

work when they stated that during growth there is a progressive solubilisation of initially insoluble hemicellulosic material, chemically similar to the water-soluble gums, from the cell-walls of the endosperm, thus accounting for the increased yields of water-soluble material. These workers provided indirect evidence of the solubilisation of initially insoluble material. Enzyme-inactivated barley extracted with alcoholic soda caused an approximately three-fold increase in the amount of mixed glucosan and pentosan extractable by cold water without, however, any notable change in the proportions of pentosan present; this is clear evidence of an initially insoluble reserve of these materials. However, Meredith et al. (1951) were of the opinion that part, at least, of the malt gum was derived from the soluble polysaccharides of barley. The evidence of Preece and Mackenzie (1952), on the other hand, indicates that a major part of the initially soluble barley gum is non-recoverable in the malt, as a result of the degradative action which has occurred.

Hall et al. (loc. cit.) found that the total pentosans decreased during the first day of steeping, the loss being attributed to dissolution of hemicelluloses into the steeping water. After steeping, the quantity of pentosan increased slowly until the green malt was kilned, when it remained approximately constant. Although the general trends in the behaviour were similar for the two varieties, Plumage-Archer and Carlsberg, one point of difference arose. Whereas in the Plumage-Archer barley and malt the contents of araban and xylan were found to be approximately equal, in the Carlsberg malt the content of araban was only 75-80% of that of the xylan, although the barley contained the same amount of araban and xylan, a state of affairs which persisted until the late stages of flooring /

flooring. This seemed to suggest that arabinose residues were preferentially removed from the hemicelluloses during the malting of this variety although their fate is, at present, unknown. These results for increased yields of pentosan on malting were explained by Preece and Mackenzie (1953), who said that the solubilisation effect quickly surpassed the degradative effect.

It will have been observed that there has been an ever-present consciousness of the part played by the cell-wall materials in the modification of malt and many attempts have been made to link various aspects of this to an "index of modification", e.g. by Fink and Hartmann (loc. cit.), Piratzky and Wiecha (1937). However, Hopkins and Norris (1935) pointed out that the changes in character and quality of the various enzymes functioning during growth, the cell-wall dissolution and the production of soluble pentosans, could all vary in rate relative to one another in different barleys and under different conditions of malting. Despite this, subsequent attempts concentrated solely on one aspect. It is now well-known that the viscosities of extracts from germinated barley decrease as growth proceeds, this effect being ascribed e.g. by Preece (1948, 1950) to breakdown of hemicelluloses and water-soluble gums, and to possible degradation of other substances such as salt-soluble proteins by Meredith and Sallans (1943). These latter authors suggested the existence of influences due to varietal and cultural conditions, as did Helm (1939), who also adduced evidence that viscosity might be influenced by conditions of malting. More recent attempts have tended to concentrate on viscosity, the most prominent being the work of Feys (1951) and Kleber et al. (1952a and b).

A major advance was made in this direction by Preece et al. (1953a /

(1953a and b), who employed autolysis relationships, but the disadvantages of this method are that the curves obtained are profoundly influenced by β-glucosan and its behaviour (obscuring pentosan changes), and that the method can of its very nature take no account of the potentialities of the grain for producing further enzymes during the periods of steeping and growth. Van Roey and Hupé (1955), who also employed autolysis techniques with some success, concluded that modification during malting depends both on gum content (and complexity) and on gum-splitting potentialities during growth. High gum content, they stated, may be no disadvantage if gum-splitting or cytoclastic enzymes are abundant. Here again, however, the results are obscured by β-glucosan. There is a further complication, as stated by Preece (1951), that it is not so much the amount of barley gum present as the form in which it exists that gives an indication of the malting quality of a barley.

Massart and van Sumere (1955) proposed a somewhat different, but with what has been said a very significant approach concerning hemicellulose solubilisation and gum complexity. The method employed was that described by Preece et al. (1950), determinations on a range of barleys being made of hemicelluloses precipitable from alkaline extracts by Fehling's solution alone (S_1-fraction) and by Fehling's solution plus acetone (S_2-fraction). Employing the formula $100(2S_1 + S_2)$, they obtained a relationship between the malting qualities of the barleys as assessed by an independent expert and the values given by substitution in the equation. Those with the lowest score represented the best malting barleys. These workers very wisely emphasise the danger of attempting to assess maltability on a single character.

A /

A consideration of the degree of degradation of hemicelluloses will reveal still more complications. To a useful extent the degree of solubilisation can be assessed but it is not yet possible to say how complete is the concomitant degradation. Water-soluble β-glucosan has been shown to be virtually eliminated during malting but the nature of the end-product is not known; with the araboxylan, the molecules are certainly less complex than they were before. However, there is a difference of opinion as to whether malt contains any free pentoses, but xylose and arabinose have been reported in malt by MacLeod et al. (1953) and Harris et al. (1956). It is profitless to argue whether these sugars are normal constituents of malt and wort because the amounts present will depend upon working conditions. In any case, the amounts of pentoses occurring free are so small as to suggest either that degradation of araboxlyan occurs only to a limited extent or that degradation products are immediately employed in transglycosylation reactions for synthetic purposes. The ultimate balancing of pentosan degradation by synthetic reactions was suggested by Fink (loc. cit.)

EXPERIMENTAL.

Materials.

Samples were taken at daily intervals during the commercial malting of Proctor barley (1955 season), a variety which, judging from its favourable characteristics, will become increasingly popular for malting. In all instances the unit quantity for determinations has been 1,000 corns, so that the figures shown may be taken as indicating the actual changes in the grain from day to day.

The barley was steeped in water for 65 hr. at 12°C., the steep liquor /

liquor being changed every 24 hr., and after draining the barley was allowed to remain in the cistern for 24 hr. of "dry steeping" before turning on to the floor. This must be regarded as part of the 8-day germination period, during which the temperature on the floor rose to a maximum of 20°C. by the fifth day. The first kiln sample was taken after 12 hr. at 63°C., whilst the finished malt had been cured for 20 hr. at 88°C. A further two commercial samples, an Ymer barley (1955 season) and its finished malt, are included in the investigation.

Extraction of initially-water-soluble hemicelluloses.

The extraction technique employed was similar to that of Preece and Mackenzie (1952) and followed enzyme inactivation. For the raw barley samples, this inactivation was obtained by refluxing the ground grain for three successive half-hour periods with 200 ml. of 80% (v/v) ethanol. On the other hand, with these samples which were wet and, consequently, could not be milled for fear of autolysis, the corns were refluxed for two successive half-hour periods with 200 ml. of 88% (v/v) ethanol to compensate for the additional water associated with these samples, the grain being added to the boiling alcohol to minimise the danger of autolysis. This was followed by refluxing for two further half-hour periods with 100 ml. quantities of 80% (v/v) ethanol. After hot-filtration the grain residues were allowed to dry in air at room temperature. In addition to inactivating enzymes this treatment removed free sugars, lipoids, amino acids, alcohol-soluble proteins and denatured coagulable protein. The dry samples were then ground, where appropriate, and extracted with water at 40°C. for three successive half-hour periods, 150 ml. of water being employed for each extraction. After centrifugation, the extracts were decanted and /

and filtered bright through Kieselguhr.

Precipitation of initially-water-soluble hemicelluloses.

These were recovered from the combined extracts by a modification of the method employed by Preece and Aitken (1953). The extracts were concentrated to 100 ml. and 50 ml. of mixed Fehling's solution added; in one instance only a precipitate was obtained at this stage and it was removed and processed separately. In all other instances precipitation occurred only on the addition of 60 ml. of acetone; the precipitate was centrifuged at a slow speed which facilitated ease of dissolution on the subsequent addition of normal hydrochloric acid to the precipitate, after which the gums were re-precipitated by the addition of acetone to 60% (v/v) concentration. Drying of the gums was effected by slowly increasing the strength of the acetone and finally washing with 95% (v/v) ethanol. The precipitate, which should now be acid-free, was collected on a tared IG4 sinter-glass crucible and dried to constant weight at 100°C.

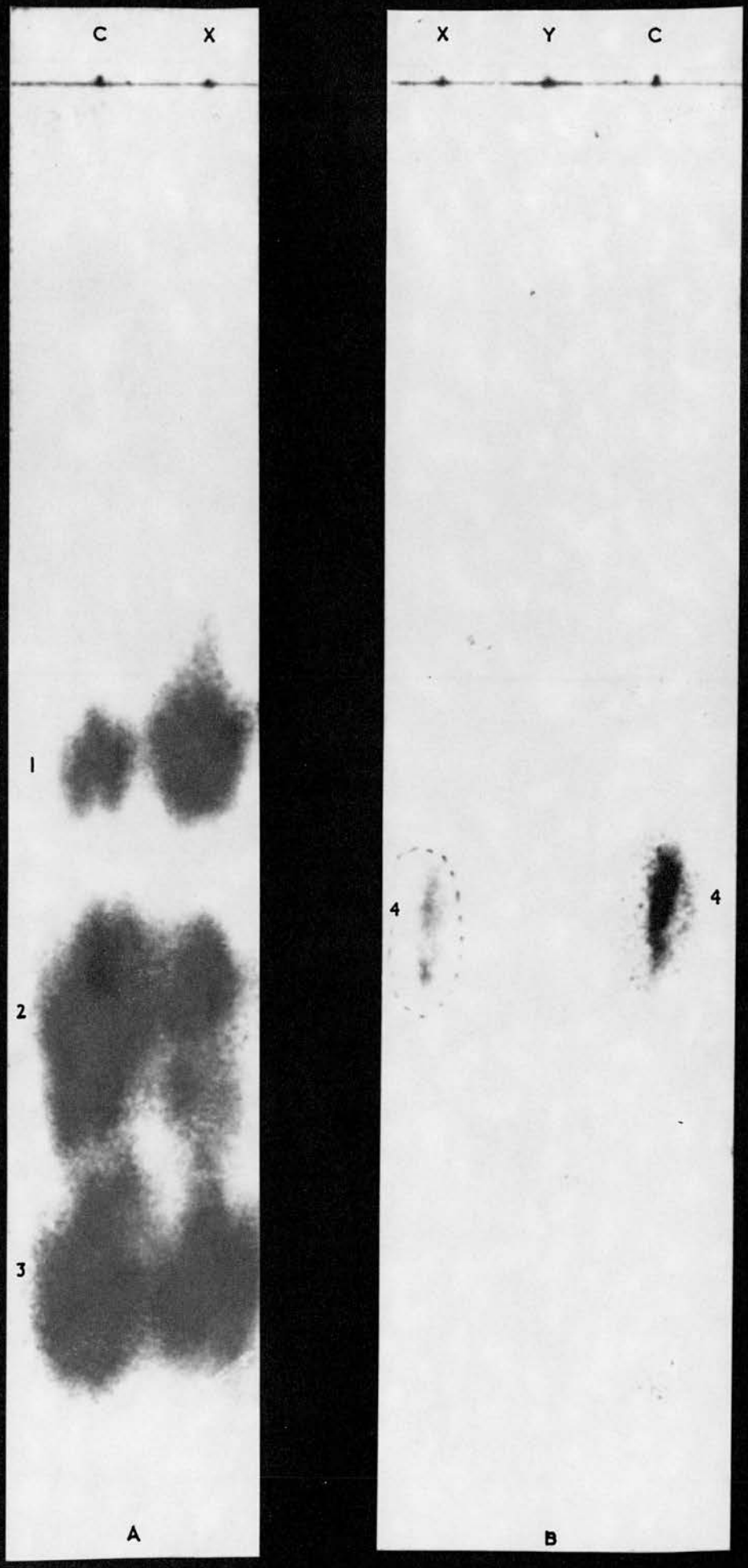
Examination of initially-water-soluble hemicelluloses.

These white, pulverulent materials were hydrolysed by boiling with 1.0 N. sulphuric acid (ca. 20 ml. per 20 mg.) for 3½ hr. under reflux. The cooled hydrolysates were neutralised with 1.0 N. sodium hydroxide solution, using methyl red as indicator, the salt formed in this neutralisation being removed by precipitation with four volumes of 95% (v/v) ethanol. The sodium sulphate was filtered off and the filtrates evaporated to dryness because it has been found that the above addition of ethanol to 80% (v/v) concentration does not effect a complete removal of salt, the amount remaining being sufficient to interfere with separation on chromatograms (vide infra). Therefore, after evaporation to dryness /

dryness, the residue was treated with 60% (v/v) ethanol (2-3 ml.) and the undissolved salt removed by filtration through a micro-filter at the pump. For the estimation of the component sugars in these solutions the technique employed was paper partition chromatography by the method described by MacLeod (1951). The chromatograms were run on Whatman No. 1 (3 mm.) paper and were irrigated for 4 days by the upper layer of the system butanol/ethanol/water (45:5:50), since this solvent effected a very good separation of arabinose and xylose. The use of the thicker paper is desirable because a greater volume of the test solution can be applied and, consequently, the accuracy of the method is increased by dint of greater titration figures in the Somogyi (1945) micro-method. Each test solution was run in duplicate and these determined separately (see Table IV). The chromatograms were developed by spraying with aniline oxalate. Plate I shows the presence of fructose in the hydrolysates of samples obtained from grain in the early stages of malting and its absence from finished malt. This will, therefore, slightly increase the apparent araban : xylan ratio.

Preparation of malt α-amylase.

100 gm. of Ymer malt (1955 season) were ground, added to 300 ml. of distilled water and extracted for 1 hr. at room temperature with constant stirring. The extract was filtered water-bright, allowed to autolyse overnight (16 hr.), then heated at 70°C. in the presence of 0.2% calcium acetate for 15 minutes. After cooling and removal of coagulated material by centrifugation, the extract was dialysed for 3 days against running water. The α-amylase was precipitated by the addition of four volumes of acetone and the enzyme taken to dryness by slowly increasing the strength of the acetone/


C
X
1
2
3
A
X
Y
C
4
4
B

acetone.

Action of α-amylase on β-glucosan and araboxylan.

Since it was intended to employ this enzyme preparation in the removal of α-glucosan from the water-soluble gums and the grains from which these gums had been obtained, it was necessary to determine the extent, if any, of the action of the α-amylase preparation on β-glucosan and pentosan substrates.

In the first instance, the effect of the preparation on β-glucosan was examined viscometrically, employing the reaction mixture, 8 ml. of 0.6875% (w/v) β-glucosan solution, 1 ml. of acetate buffer pH 4.6 and 2 ml. of enzyme stock solution (2 mg. per ml.). The reaction was carried out at 25°C. and there was no detectable decrease in viscosity over a period of 2 hr., as shown in Figure 9, which also includes the result of a similar experiment with rye araboxylan as substrate.

It was then necessary to determine if free sugars were produced by the action of the α-amylase preparation on β-glucosan and araboxylan under normal, de-starching conditions. In this series, reaction mixtures similar to the above were employed with the addition of concurrently-run controls of the β-glucosan and araboxylan substrates and the enzyme preparation. Where appropriate, 2 ml. of α-amylase stock solution were added to the contents of the tubes, already at a temperature of 65°C. After 2 hr. a further 2 ml. of enzyme solution were added and the treatment continued at 65°C. for 2 hr. The reactions were terminated by heating the tubes in boiling water for five minutes and, after cooling, four volumes of 95% (v/v) ethanol were added to each reaction mixture. The supernatant liquors obtained on centrifugation were evaporated to a low volume and examined chromatographically, the /

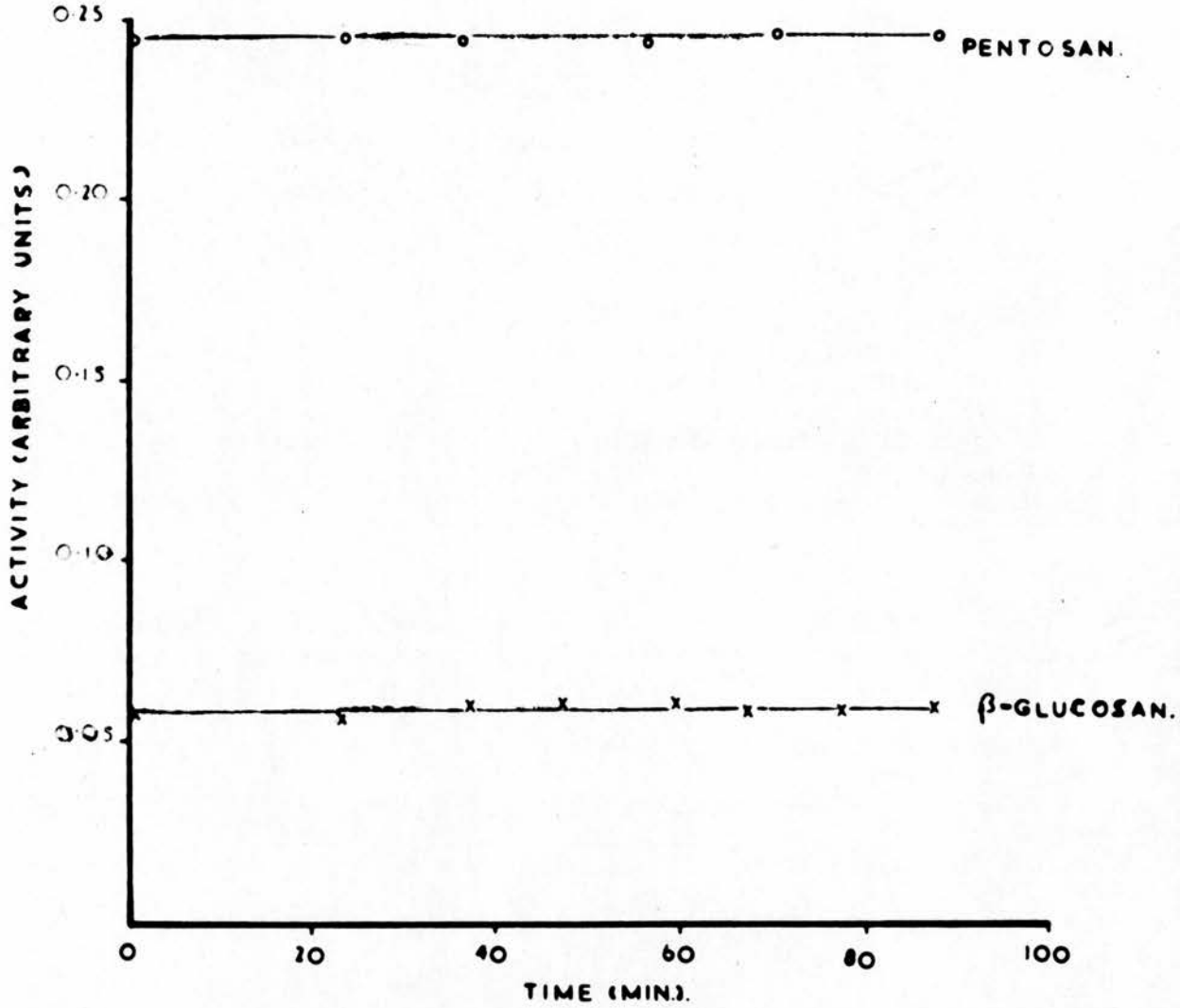


Figure 9.

The effect of the malt α-amylase preparation on the viscosities of aqueous solutions of pentosan (araboxylan) and β-glucosan.

the irrigating solvent being butanol/ethanol/water (45:5:50). The chromatograms were run for 3 days and, after developing with aniline oxalate, it was seen that there was a slight pink colouration associated with the α-amylase control in the region of the starting line and a similar effect was noticed with the β-glucosan and araboxylan reaction mixtures, although absent from the control reactions with these two substrates. A quantitative determination of the carbohydrate associated with this preparation showed that 47% of the dry weight consisted of carbohydrate. The results of this investigation are shown on Plate II.

From the latter series of experiments, although no free sugars have been detected, there is the possibility that the enzyme does split internal linkages in the polysaccharide chain. Therefore, to check this, a 0.5% (w/v) stock solution of a rye araboxylan (contaminated with 3-4% of glucosan, presumably β-glucosan) was employed in a reaction mixture consisting of 10 ml. of this stock solution, 1 ml. of acetate buffer of pH 4.6 and 2 ml. of enzyme stock solution, the reaction being run at 65°C. for 3 hr., after which a further 2 ml. of enzyme solution were added and the reaction continued for 3 hr. Pentosan and α-amylase controls were run under the same conditions, and the reactions were terminated by raising the temperature to 100°C. for five minutes. After cooling, the increase in reducing power was measured by the Somogyi (1945) micro-method. From these determinations it was found that the increase in reducing power is equivalent to 0.77 ml. of 0.005 N. sodium thiosulphate which, calculated as xylose, corresponds to a 0.21% increase in reducing power or to the splitting of one linkage in every five hundred in a xylan chain.

It was necessary to determine if this increase represented any loss /

PLATE 11.

Chromatogram to show the absence of action by the α-amylase preparation on β-glucosan and pentosan substrates.

(Solvent, butanol: ethanol: water).

C. - control.

G1. - β-glucosan reaction mixture.

P1. - pentosan reaction mixture.

E. - enzyme control.

G2. - β-glucosan control.

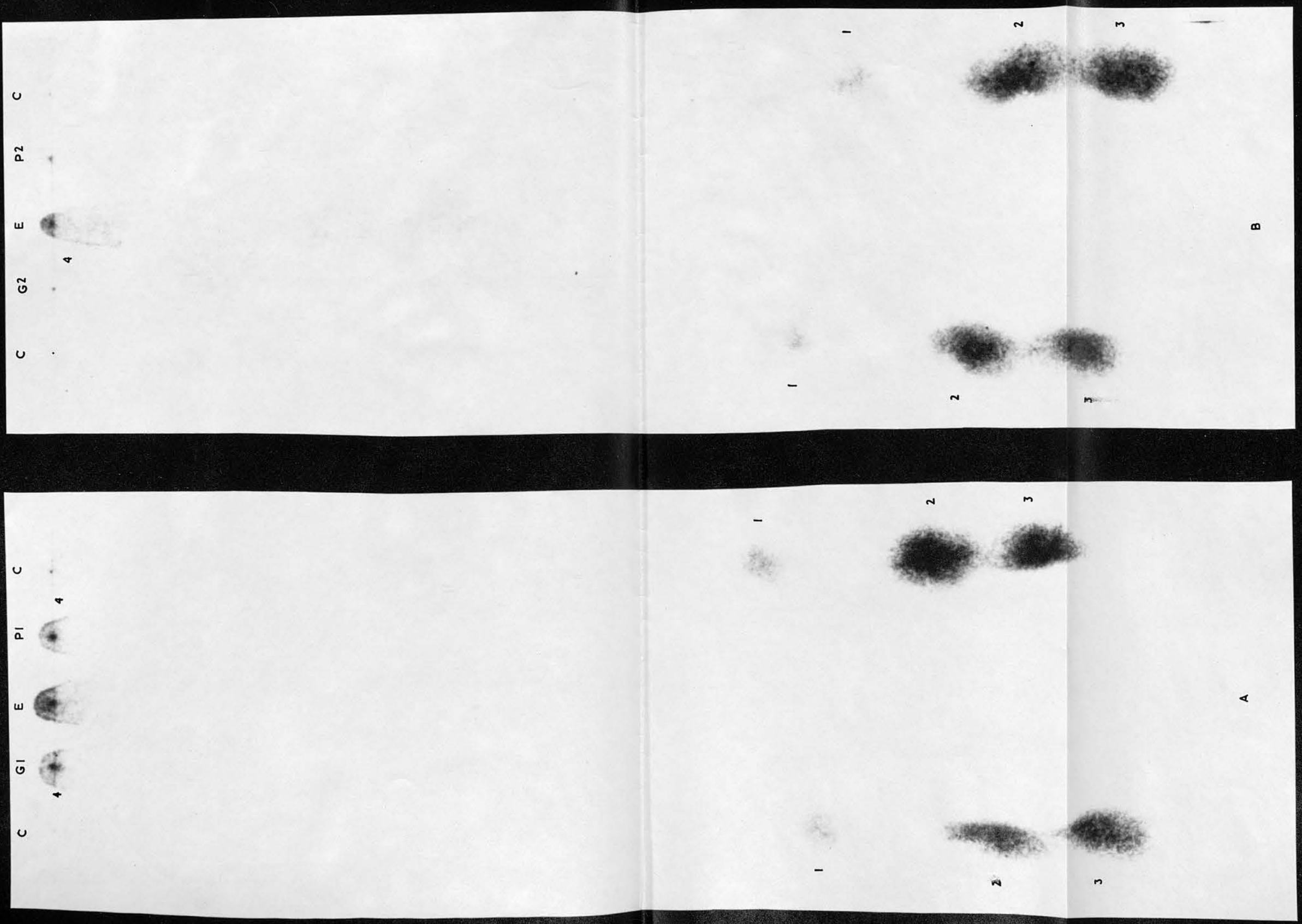
P2. - pentosan control.

1. - glucose.

2. - arabinose.

3. - xylose.

4. - high-molecular pentosan.


C
G2
E
P2
C
4
B
1
2
3
C
GI
E
PI
C
4
A
1
2
3

loss of precipitability of the pentosan. The reaction mixture and conditions were similar to those employed in the last series, but the control, in this instance, involved adding 4 ml. of the α-amylase stock solution whilst the substrate was being heated at 100°C. to ensure that no reaction occurred. Heating at 100°C. was extended to ten minutes and, after cooling, four volumes of 95% (v/v) ethanol were added, the precipitates centrifuged, taken to dryness and finally collected on sinter-glass crucibles (IG4) which had been heated to constant weight. The gums were then heated at 100°C. until weight-constancy was attained. The results are tabulated below:-

	Weight gum (gm.)
Reaction mixture	0.0370
Pentosan control	0.0370

Clearly, from these results it can be concluded that there is no loss of precipitability by the pentosan substrate at 65°C. in the presence of this α-amylase preparation, and taken overall, the action of this preparation on β-glucosan and araboxylan is, at the very most, negligible.

Treatment of initially-water-soluble hemicelluloses with α-amylase.

Having established the absence of significant action by the α-amylase preparation on β-glucosan and araboxylan substrates, removal of the α-glucosan from the water-soluble hemicelluloses was effected. In each instance, 50 mg. (dry weight) of gum were dissolved in 10 ml. of water to which, after cooling, 1 ml. of acetate buffer pH 4.6 was added and the temperature raised to 65°C. 2 ml. of α-amylase stock solution (2 mg. per ml.) were then added and the reaction run for 3 hr. at 65°C., after which a further 2 ml. of enzyme solution were added and the treatment continued for a

further /

further 3 hr. The reaction mixtures were then heated to 100°C. for five minutes and, after cooling, the solutions were dialysed for 2 days. The gums were then recovered by precipitating with four volumes of acetone, taken to dryness and collected on a sinter-glass crucible (IG4).

The recovered gums were then analysed quantitatively for the component sugars glucose, arabinose and xylose by the method of MacLeod (loc. cit.). It is significant that the ratio of arabinose : xylose underwent no significant change as a result of the α-amylase treatment. The results are shown in Table IV.

De-starching of grains.

The grain residues of water-extraction were subjected to α-amylase treatment with a view to removing associated starch. The grains were mixed with a suitable volume of water, sufficient to avoid a thick paste, and the mixture was heated to boiling for a few minutes, after which the whole was placed in an autoclave. The pressure was raised to and maintained at 12 lbs. per square inch for 1 hr. and, after removing from the autoclave, the temperature of the mixture was brought to 65°C. when approximately 10 ml. of the α-amylase stock solution were added. This treatment was continued for approximately 3 hr. during which the breakdown of starch previously gelatinised by autoclaving was followed by noting the colour produced by adding iodine solution to a test portion. After this 3-hr. period the temperature of the mixture was raised to boiling and the autoclaving repeated. This was necessary because of the very resistant nature of the starch granules, but complete gelatinisation was effected after a total of 4 × 1 hr. periods in the autoclave. In the final stages of this de-starching process it was found that testing was rendered more reliable if,

after /

after addition of iodine solution to a test portion, microscopical examination was included.

When these tests indicated the absence of starch, the grain residues were filtered off at the pump, boiled with two successive portions of water and filtered. The material was then washed with further portions of hot water until approximately one litre of combined filtrates had been collected. These filtrates were reserved for further treatment and residues were dried by heating at 100°C.

Extraction of initially-water-insoluble hemicelluloses.

The dry, starch-free material was extracted at room temperature with 150 ml. of 4% (w/v) caustic soda solution for ½ hr. with continuous stirring and, after centrifugation, the residues were subjected to 2 × ½ hr. extractions with 75 ml. portions of caustic soda solution. The combined alkaline extracts were then filtered bright through a pad of paper pulp which had been prepared by treating Whatman No. 1 filter paper with a suitable volume of 4% (w/v) caustic soda solution. Filtration complete, washing of the pad was kept to the very minimum.

Precipitation of initially-water-insoluble hemicelluloses.

A volume of mixed Fehling's solution, corresponding to half the volume of the combined filtrates, was added slowly, with stirring. No precipitate was obtained at this stage (S_1 complex) and so acetone was added slowly to 40% (v/v) concentration when precipitation took place. After centrifugation, the precipitate (S_2 complex) was dissolved in 1.0 N. hydrochloric acid and by the addition of acetone to 60% (v/v) concentration the hemicelluloses were re-precipitated, taken to dryness by slowly increasing the strength of acetone and collected on a tared IG4 sinter-glass crucible /

crucible to be heated to constant weight at 100°C.

The preparations obtained thus were analysed by the method of MacLeod (loc. cit.) for the component sugars glucose, arabinose and xylose as above. Chromatographic analyses revealed the presence in the hydrolysates of material believed to be uronic acid in nature (Plate III).

Treatment of initially-water-insoluble hemicelluloses with α-amylase.

This procedure was similar to that discussed for the removal of α-glucosan from the initially-water-soluble hemicelluloses and the results in Table V show the compositions of these alkali-soluble materials.

Material soluble at 100°C.

On completion of de-starching, the mother liquors and the washings of the grain residues were reserved for further treatment. These were dialysed for 4 days and, after filtering bright through Kieselguhr, two volumes of acetone were added. These precipitates, which were taken to dryness in the usual manner, were of a fibrous nature early in the malting process but this gradually gave way to a pulverulent character as modification neared completion.

Examination of these materials.

In each instance a 20 mg. (dry weight) sample was hydrolysed by 20 ml. of 1.0 N. sulphuric acid for 3 hr. under reflux and the solution, after cooling, was made up to 25 ml. with distilled water. 2.5 ml. aliquots were withdrawn for the estimation of the total reducing power, neutralised with 1.0 N. sodium hydroxide solution to the colour change of methyl red and the usual Somogyi (1945) micro-method applied. The results were calculated in terms of glucose equivalents. The remainder of the hydrolysate was then treated according to MacLeod (loc. cit.) and the component sugars estimated /

PLATE 111.

Chromatogram to show the presence of uronic acids in hydrolysates of initially-water-insoluble hemicelluloses.

(Solvents, (A) - butanol: ethanol: water,

(B) - butanol: acetic acid: water).

C. - control.

X. - sugars obtained from acid hydrolysate.

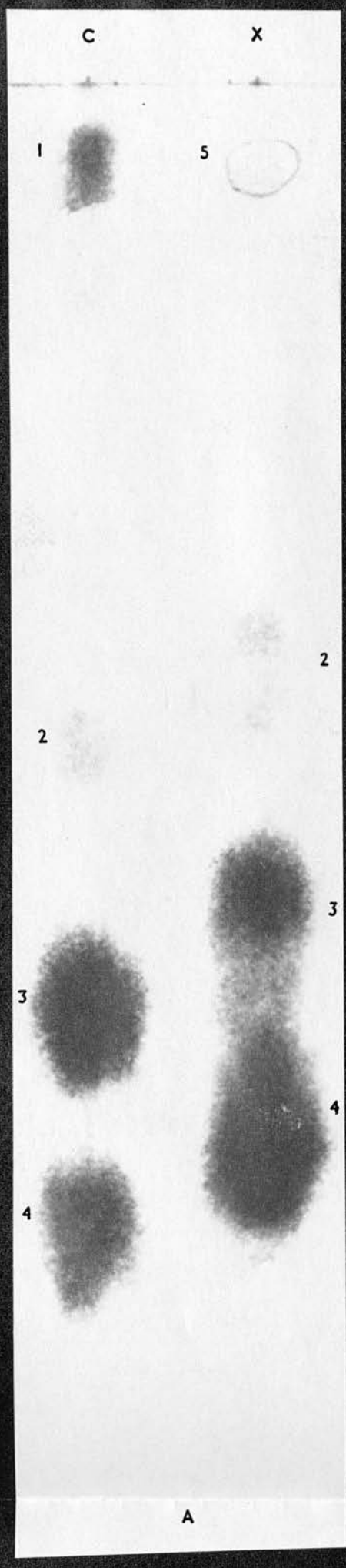
1. - galacturonic acid.

2. - glucose.

3. - arabinose.

4. - xylose.

5. - glucuronic acid?


C
X
1
5
2
2
3
3
4
4
A

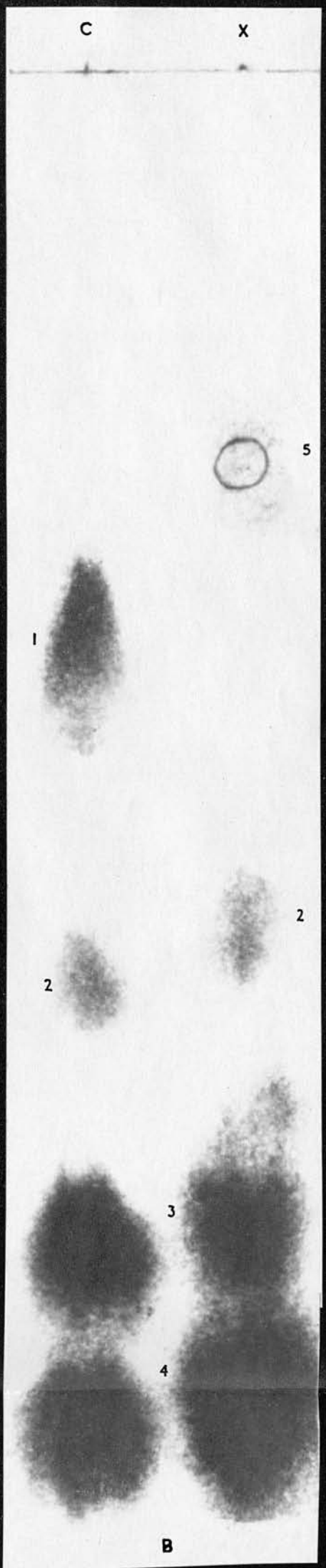

C
X
5
1
2
2
3
4
B

TABLE IV.

Composition of the Initially-Water-Soluble Hemicelluloses Obtained from Samples Taken During the Malting of Proctor Barley.
(Results shown in mg. per 1,000 corns)

Stage	Time (hr.)	Total gum	Total glucosan	α-glucosan	β-glucosan	Araban	Xylan
Raw barley	-	126	95	34	61	14	17
Steep 1	65	115	87	23	64	16	12
2	89	140	105	32	73	15	19
Floor 1	24	172	129	41	88	29	14
2	48	270	203	89	114	52	16
3	72	360	267	107	160	48	44
4	144	455	373	180	193	62	20
5	168	322	256	109	147	45	30
Kiln 1	12	310	237	142	95	36	36
2	42	146	37	17	20	44	65
Ymer raw barley	-	231	182	14	68	32	16
Kiln	59	117	13	2	11	41	63

TABLE V.

Composition of the Initially-Water-Insoluble Hemicelluloses Obtained from Samples Taken During the Malting of Proctor Barley.
(Results shown in mg. per 1,000 corns)

Stage	Time (hr.)	Total hemicellulose	Total glucosan	α-glucosan	β-glucosan	Araban	Xylan
Raw barley	-	1,472	561	241	320	489	421
Steep 1	65	1,242	323	0	323	322	596
2	89	1,270	382	110	272	341	547
Floor 1	24	1,363	423	146	277	434	506
2	48	1,063	146	-	-	311	606
3	72	1,261	340	170	170	368	552
4	144	1,097	232	82	150	316	550
5	168	1,116	187	74	113	338	590
Kiln 1	12	962	174	62	112	252	535
2	42	595	154	76	78	109	332
Ymer raw barley	-	1,418	563	-	-	428	426
Kiln	59	1,000	95	0	95	263	643

TABLE VI.

Percentage Recovery of Carbohydrate from Mother Liquors of De-Starching Treatment.
(Results shown in mg. per 1,000 corns)

Stage	Time (hr.)	Total material	Weight taken for hydrolysis	Glucosan	Araban	Xylan	Percentage recovery
Raw barley	-	2,238	20.1	10.5	4.9	1.3	83.0
Steep 1	65	-	19.8	10.5	5.2	1.6	87.3
2	89	-	20.4	11.2	4.9	1.4	85.7
Floor 1	24	2,122	-	-	-	-	-
2	48	1,680	19.8	11.1	5.2	1.1	87.9
3	72	2,333	21.7	14.2	2.8	2.2	88.6
4	144	1,553	20.5	13.1	3.6	1.3	87.8
5	168	-	20.5	14.3	2.2	1.4	87.2
Kiln 1	12	2,112	20.8	13.1	4.9	0.9	90.8
2	42	1,130	20.0	12.0	4.4	1.5	89.5
Ymer raw barley	-	1,515	19.7	10.6	3.8	1.8	82.5
Kiln	59	672	22.3	14.1	2.2	2.2	82.4

estimated quantitatively. By combining these two determinations it was thus possible to calculate the percentage recovery of carbohydrate as shown in the specimen calculation below. The results of these determinations are shown in Table VI.

Specimen Calculation of Percentage Carbohydrate Recovery.

Sugar	Conversion Factors	Anhydro Sugar	ml. 0.005 $Na_2S_2O_3$	mg. polysaccharide
Glucose	0.135	0.121	18.64	18.64 × 0.121
Arabinose	0.148	0.130	0.48	0.48 × 0.130
Xylose	0.137	0.120	0.92	0.92 × 0.120
			20.04	

But total glucose equivalent (for 21 mg.) = 143.9 ml.

$\therefore$ Glucosan = $143.9 \times \frac{18.64}{20.04} \times 0.121$ mg. = 16.19 mg.

Araban = $143.9 \times \frac{0.48}{20.04} \times 0.130$ mg. = 0.44 mg.

Xylan = $143.9 \times \frac{0.92}{20.04} \times 0.120$ mg. = 0.79 mg.

i.e. a total of 17.42 mg.

Hence percentage recovery = $\frac{17.42}{21} \times 100$

= 82.9

DISCUSSION.

The effect of malting upon β-glucosan is shown in Figure 10. The amount of water-soluble β-glucosan rises steadily from a low value in barley (about 60 mg. per 1,000 corns) to a maximum at the sixth day of growth (190 mg.), rapidly falls on the seventh and last day of flooring and during kilning to a very low final figure (20 mg.). It is significant that β-glucosan has not been completely eliminated and, therefore, the present results are in agreement /

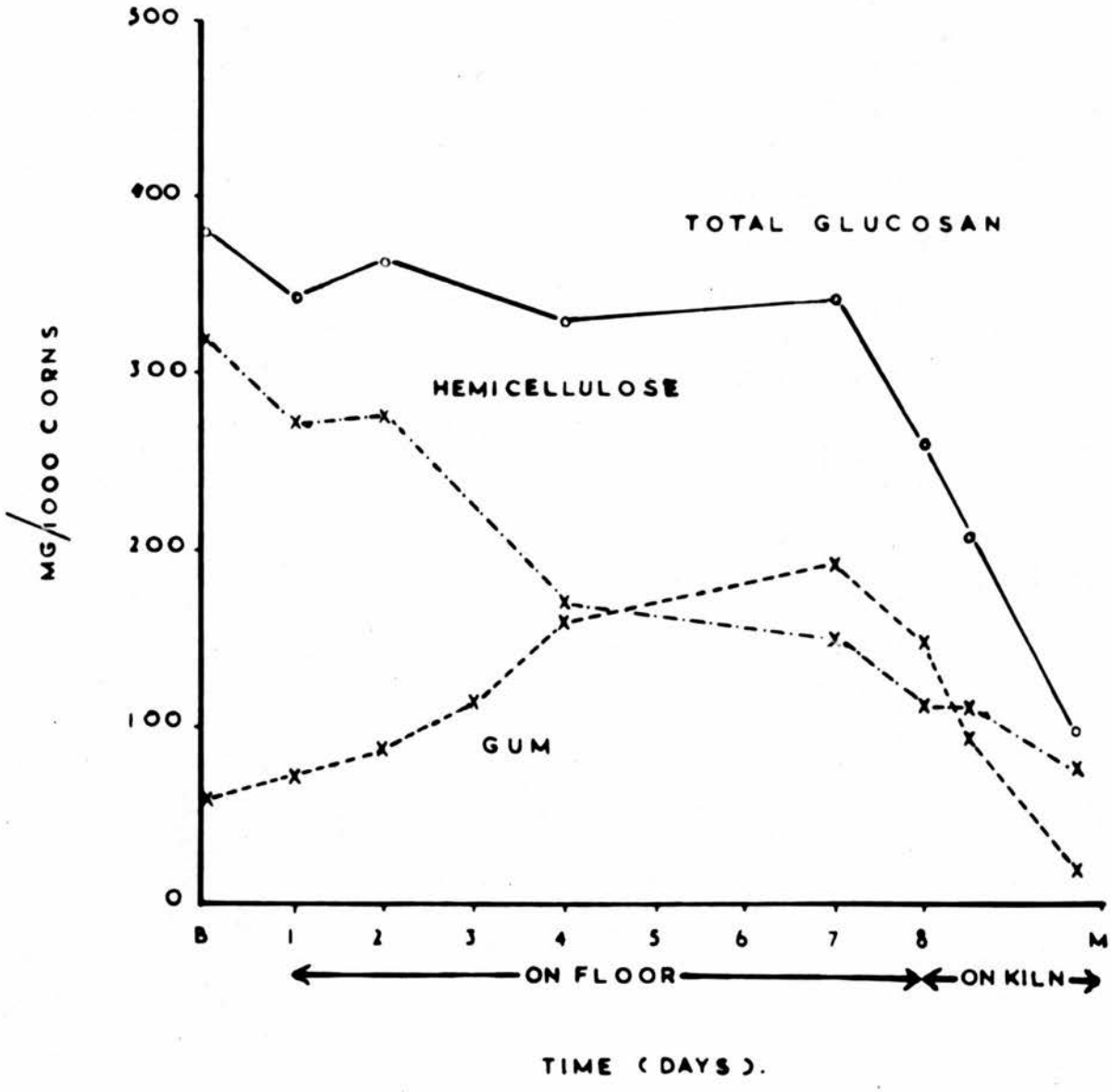


Figure 10.

The amounts of recoverable water-soluble and alkali-soluble β-glucosan obtained from grain during the commercial malting of Proctor barley.

agreement with the observations of Preece and Mackenzie (1952), who found that the yield of β-glucosan from finished malt was very small but only recoverable at the higher salt concentration, 50% (w/v) ammonium sulphate. It is probable that this β-glucosan represents very low-molecular material, perhaps the limit of endo-β-glucosanase action. Thus, the present results are contrary to the findings of Piratzky et al. (1938), who failed to detect α-amylan (now called β-glucosan) in the final malt. This residual β-glucosan, by its very nature and amount, will have little effect upon the viscosity of extracts.

The amount of alkali-soluble hemicellulose begins at a high value (320 mg. per 1,000 corns) and falls steadily in amount throughout the process, reaching 78 mg. in the finished malt. In total, β-glucosan falls very slowly up to the point when flooring is almost complete, and thereafter the drop is very much accelerated.

It is observed that initially insoluble β-glucosan decreases steadily and the water-soluble material increases during growth and, therefore, it is suggested that solubilisation of initially insoluble material overshadows the concomitant degradation, prior to kilning. The fall which occurs when the grain is kilned may be the result of raising the temperature towards and beyond the optimum of cytolytic action. In other words, degradation is the predominant factor but solubilisation has not been raised to the same level, probably because the amount of attackable material has been reduced to such a low level that the observed effect is small. Sandegren and Enebo (1952) have shown that there is a degree of resistance to heat on kilning, endo-β-glucosanase being much more stable in the grains than in solution. Thus, concerning the degradation /

degradation of β-glucosan, it is emphasised that the relative amounts of exo- and endo-enzymes in the cytolytic system are constantly changing throughout the malting process and, therefore, the breakdown of this material will differ quite significantly at the different stages. However, the available evidence does suggest that there is an insoluble reserve of β-glucosan, and, although solubilisation is thought to be a predominantly enzymic effect, the possibility of mechanical penetration by the solvent cannot be ignored completely.

The nature of the α-glucosan accompanying the water-soluble β-glucosan is not known. In this connection it should be noted that Gilles et al. (1952) have shown the presence of small amounts of a dextrorotatory, non-starchy α-glucosan in barley whilst, more recently, Peat et al. (1956) reported that such material in maize is of the nature of glycogen.

Pentosan changes during malting are small (Figure 11) and, therefore, their interpretation is rendered more difficult. Water-soluble pentosan increases irregularly from 31 mg. per 1,000 corns in barley to 109 mg. in malt. The initially-water-insoluble pentosan (910 mg. in barley) decreases slightly during steeping but attains a steady maximum (approximately 900 mg.) from the fourth day to the end of flooring, thereafter dropping with great rapidity on kilning, reaching a final value at 441 mg. per 1,000 corns. This rapid decrease in residual pentosans on kilning is surprising because the level of pentosanase activity attained during growth, as shown by the in vitro studies of Preece and MacDougall (1956), is quite low, but, naturally, a greater activity is anticipated when the temperature is raised. Furthermore, the affinity of the pentosanase system for pentosan material laid down in the intact barley /

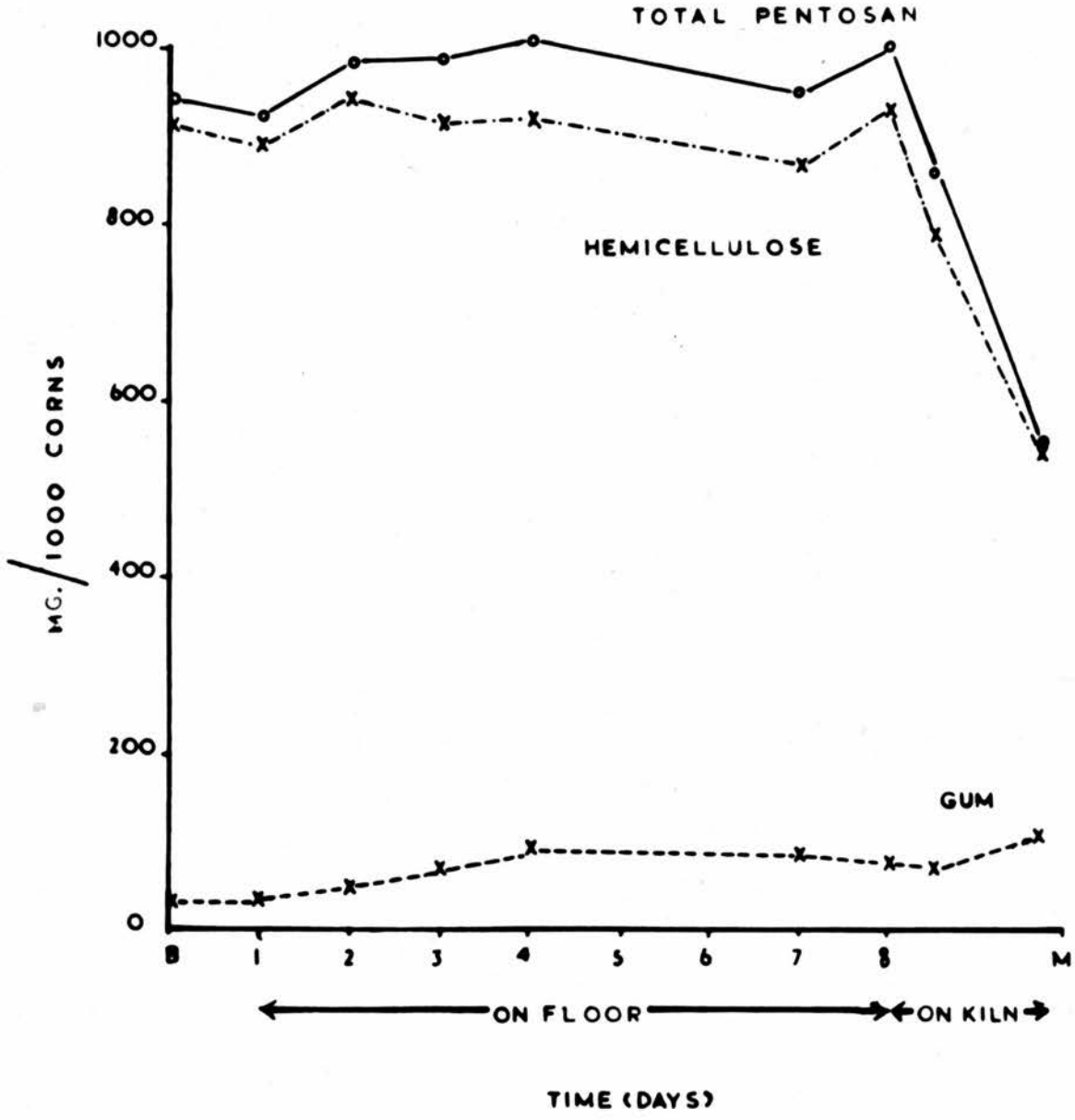


Figure 11.

The amounts of recoverable water-soluble and alkali-soluble pentosan (araboxylan) obtained from grain during the commercial malting of Proctor barley.

barley corn may be appreciably greater than for a rye araboxylan. In view of the relatively small increase in amount of soluble pentosans during growth and the relatively constant level of alkali-soluble material it is difficult to comprehend more than a minor degree of solubilisation and degradation. During kilning, degradation must be quite extensive because the great decrease in amount of residual pentosan is accompanied only by the very slightest increase in soluble material. However, an explanation based entirely upon the results from enzymic studies is out of the question. The slight increase in amount of water-soluble pentosan from barley to malt may be the result of the solubilising effect overcoming the degradative system with the possible intervention of transarabinosylation as suggested more recently by Preece and Hobkirk (1955).

A study of Figure 12 is quite revealing. The araban : xylan ratio of the water-soluble pentosan rises during flooring whilst there is a steady decrease in this ratio in the residual pentosan. This may indicate a constant, preferential removal of arabinose units from the initially insoluble material during growth, a possibility which was suggested also by Hall et al. (1955). However, during kilning the percentage of araban in the water-soluble pentosan falls with great rapidity, and, since there is some doubt whether free pentoses occur in malt, the arabinose may be utilised by the grain in some necessary metabolic process.

The arabinose figures presented in Table IV may be slightly higher than they ought because Plate I shows the presence of fructose in the hydrolysates of gums obtained from raw barley (and those taken during the early stages of growth), but fructose appears to be absent from the malt samples. The amount of fructose must /

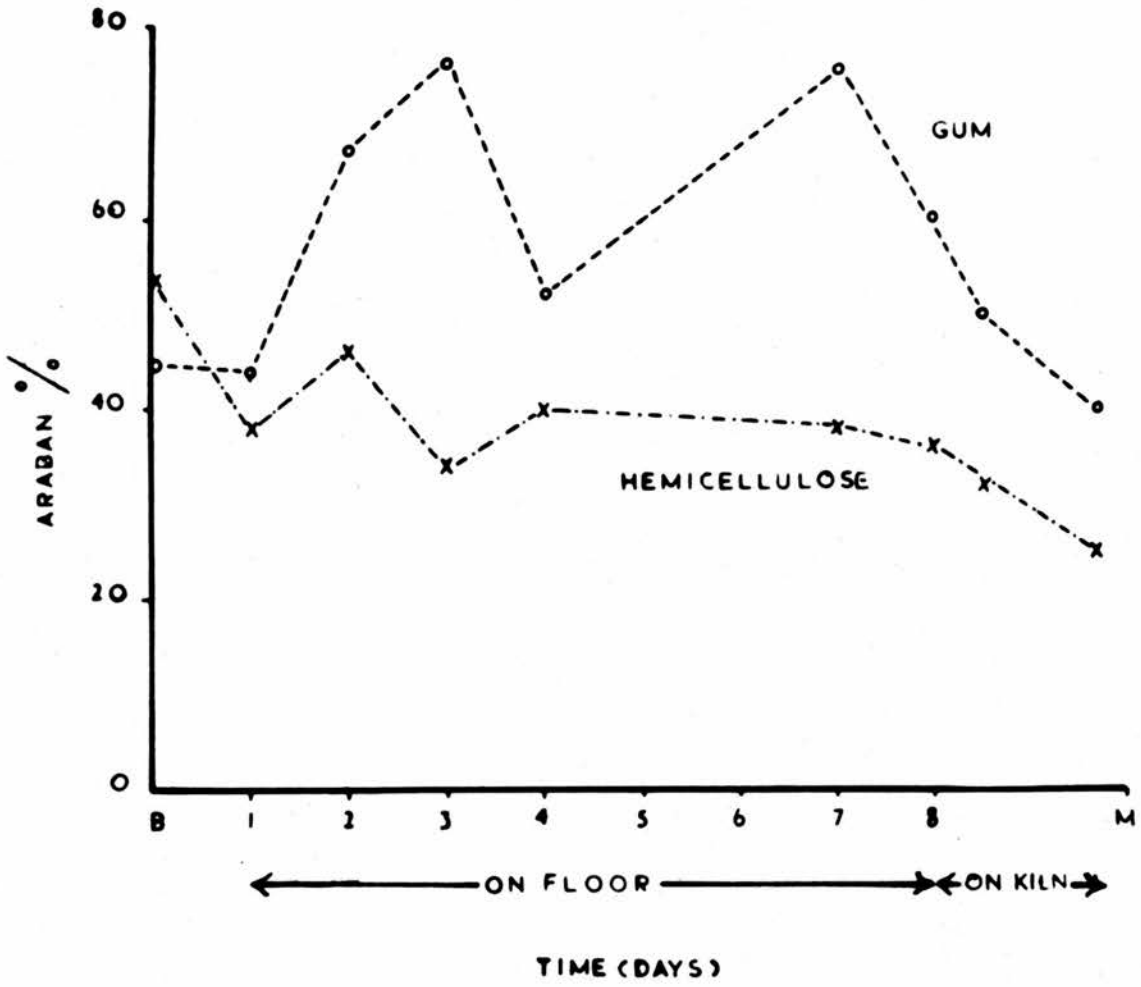


Figure 12.

Changes in the percentage araban content of recoverable water-soluble and alkali-soluble pentosan obtained from grain during the commercial malting of Proctor barley.

must be very small, its presence no doubt arising from the initial alcohol treatment of the grains which could not be broken up and therefore complete extraction by the boiling alcohol was not effected. Unfortunately there is no suitable method of correcting for these trace quantities. The presence of uronic acid residues in the hydrolysates of the alkali-soluble hemicellulose is shown in Plate II. Chromatograms were developed in two different systems, butanol:ethanol:water (45:5:50) and butanol:acetic acid: water (40:10:50), the former giving a pink spot of very low mobility which was greatly increased by the latter system. In this way, and also by comparing with concurrently-run controls incorporating glucuronic acid, the presence of uronic acid residues was confirmed.

That solubilisation of pentosans occurs is undoubted, the major problem being the determination of the factors upon which this depends. Mechanical penetration by the solvent is important and the ease with which this takes place varies from one sample of barley to another, as can readily be shown by autolysis techniques. Preece et al. (1953a and b) have stated that, from the point of view of malting modification, the most favourable conditions are those shown by a rapid rise in soluble gum to a maximum after 1-2 hr. The absence of such a property, however, can be overcome by the application of special treatment in the early stages. The present sample of Proctor barley is an example of this, the extra steeping period possibly having the result of producing a superior malt.

Preece and MacDougall (loc. cit.) have shown recently that simple aqueous extraction of ground barley at 40°C. may remove only about one-half of the pentosan which is actually water-soluble, but /

but the remainder is brought into solution during a brief autolysis period. Such incomplete extraction in the absence of autolytic enzymes may be explained by the manner in which protein is distributed throughout the grain. In other words, protein is probably masking a certain proportion of the pentosan soluble in water. If this is so, it appears that the autolytic system of enzymes possesses the power of removing this "shell" and it is not unlikely that, as growth proceeds, this protein would be removed with increasing ease as the proteolytic activity increased. In this way mechanical penetration by the solvent would be increased. This explanation is an advance on that put forward by Meredith, Watts and Anderson (1953), who, on the basis of poorly-controlled experiments, suggested that there is a protein-carbohydrate linkage in the barley grain. A papain preparation, whose action on carbohydrate substrates was not stated, was employed to remove the contaminating nitrogenous material. However, they could provide no evidence in support of the existence of chemical linkages and, therefore, it may be concluded that the mechanical extraction of barley gums is dependent upon the physical relationship of protein with carbohydrate. It is suggested, therefore, that the greater quantity of the β-glucosan and a lesser amount of pentosan may be laid down outwith a protein "shell" within which is distributed the greater part of the pentosan material, the solubilisation of the latter depending upon the removal of this ~~portion~~ PROTEIN. The assumption of such a "shell" requires its dissolution by alkali to account for the extracted material obtained in the present work.

Recently Cook (1957) stated that, during growth of barley, killing or removal of the embryo after 3 days did not affect the physical modification of the grain in any way; in fact, soluble material /

material was increased despite less enzyme activity in the finished malt than would have been present otherwise. It would appear, therefore, that excess active material is produced by the growing corn and so the possibility for need of the intervention of an "hemicellulase" is decreased. There may be sufficient enzymes present to bring about a removal of interfering protein, thus facilitating mechanical penetration by the solvent and the concomitant degradation. An explanation on the above lines would explain the rapid fall in recoverable, residual pentosan during kilning.

Degradation of pentosan to simple sugars and oligosaccharides is very slow under the influence of barley enzymes or even of those of green malt, and the most easily observed aspect of this slow change is a release of free arabinose. Under the influence of autolytic enzymes the arabinose/xylose ratios for gums and hemicelluloses show only a negligible change (Figure 13) in comparison with the profound changes occurring during malting (Figure 12). This result is not altogether surprising because autolysis techniques can, at the very most, simulate only the conditions prevalent during the earliest stages of the malting process. Solubilisation of pentosans in raw barley is dependent probably almost exclusively upon mechanical factors, whilst in growing barley solubilisation may be effected by a combined mechanical and enzymic action.

The work of Preece and MacDougall (loc. cit.), to which reference was made previously, indicated that no material could be recovered when barley grain residues, from which starch and water-soluble gums had been removed, were treated with barley extract. On the other hand, when subjected to the action of green-malt extracts and preparations, material of carbohydrate nature was recovered /

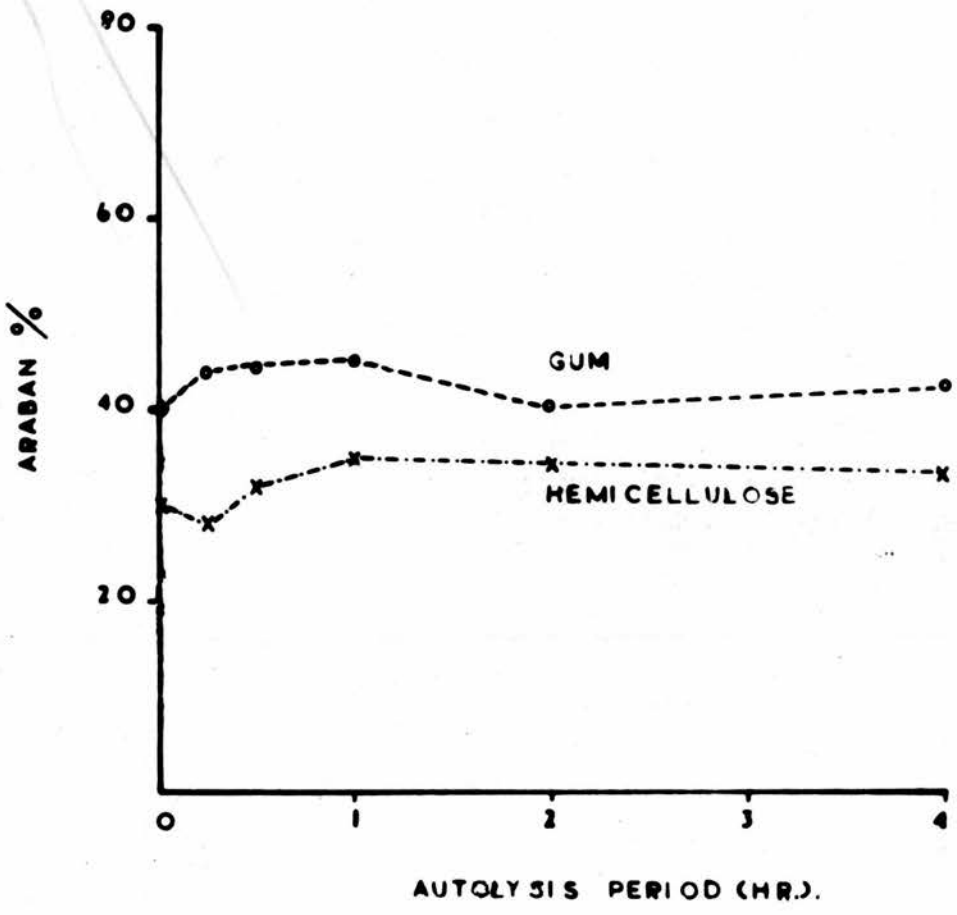


Figure 13.

Changes in the percentage araban content of water-soluble and alkali-soluble pentosan obtained during the autolysis of Ymer barley.

recovered. The amount of this material was small when the time and activity factors are considered, but it appears that pentosan solubilisation characters differ in raw and growing barley. Another significant point is that the composition of the product obtained by the action of the green-malt preparation on the residues is of approximately the same composition as the hemicellulose obtained in the present work (Table V) when barley grain residues were extracted with 4% (w/v) soda. The absence of solubilising enzymes in raw barley may explain the constant nature of the percentage araban in gum and hemicellulose preparations obtained during autolysis.

Therefore, mechanical penetration by the solvent of raw barley and of grains during the very early stages of growth appears to be the predominant factor, but in the later stages of growth enzymic action may play a part.

The origin of the malt gum remains to be established. Meredith and Anderson (1955) expressed the opinion that the malt gum may in fact owe its origin to the original barley gum. However, present evidence suggests that the initially soluble hemicellulose of malt is derived almost entirely, if not completely, from the initially insoluble material, especially in view of the great differences in amounts of recoverable alkali-soluble material from barley and malt. It is seen that these two preparations from malt (Tables IV and V) possess approximately the same percentage of β-glucosan whilst the most significant difference lies in the araban content (34 and 21% respectively), an explanation for which has been given already. It is thought that the water-soluble β-glucosan of malt is a "limit dextrin", in which case the insoluble material, also thought to be low-molecular, will undergo little /

little, if any, degradation on solubilisation.

Many attempts have been made to relate gum content alone, or in combination with endo-β-glucosanase activity of the barley, to malting quality. Such methods are of little use because too many factors are involved, of which Preece (1951) considers the form in which the gum exists to be one of the most important. The method of Massart and van Sumere (1955) was different in that barleys were scored according to the relation $100(S_1 + S_2)$. Application of this formula to the present work gives the values 147.2 and 141.8 for Proctor and Ymer barleys respectively. According to this method those barleys with the lowest score constitute the best malting samples, but in the present case, and although it is very unfair to say so on the basis of two results, agreement has not been reached because maltsters reports show that Proctor has superior malting qualities. It is realised that to compare two barleys and then set out a method of assessing malting potential is unwise, but a study of Tables IV and V may provide an explanation of the superiority of Proctor. Ymer barley yielded less total soda-soluble hemicellulose than Proctor whilst, in the finished malts, Proctor possessed only approximately one-half of the material obtained from Ymer. The total water-soluble gums obtained from Proctor barley were approximately one-half of the amount extracted from Ymer, but in the finished malts these two varieties possessed almost equal amounts of these gums. This would appear to indicate that solubilisation and degradation of hemicellulosic material had been more complete in the malting of Proctor barley, i.e. better modification has been achieved of these materials. However, it must not be forgotten that more than one factor is involved in determining the malting quality of a barley. The superior malting properties /

properties of Proctor were shown also in some work by Hall et al. (1954), in which they found that the malting of Proctor and Spratt-Archer barleys under the same conditions resulted in a Proctor malt with a superior degree of modification of starch and cell-wall carbohydrates; in fact, there was a suggestion of over-modification.

In Table VI are shown figures for the percentage recovery of carbohydrate from the mother liquors of the de-starching treatment. These materials are of approximately similar composition throughout malting, but it is difficult to explain their occurrence. It is possible that extraction at 40°C. has resulted in only partial removal of the water-soluble gums, a situation encountered by Preece and MacDougall (loc. cit.). Alternatively, material soluble at temperatures greater than 40°C. may be present in grains. Solubilisation of initially insoluble hemicelluloses by the malt α-amylase preparation employed in the de-starching treatment can be ruled out on the basis of results of control experiments.

SUMMARY.

There is extensive solubilisation and degradation of β-glucosan during malting, but a small significant amount is recoverable from finished malt. From the available evidence it appears that the solubilisation of this material is a predominantly enzymic effect with only a small contribution by mechanical factors.

Water-soluble pentosan increases slightly during the conversion of barley to malt, whilst solubilisation of pentosans during kilning is very extensive. These facts cannot be accounted for by the enzymic effect alone. It is considered that solubilisation of pentosans is a predominantly mechanical action plus a small pentosanase effect. Whilst this may be the explanation in the later stages of malting, solubilisation of pentosans in raw barley is thought to be dependent entirely upon mechanical penetration by the solvent.

A mechanism for pentosan solubilisation has been suggested .

It appears that malt gum is derived almost entirely from initially-water-insoluble hemicellulose.

No satisfactory assessment of malting quality can be made from the determination of initially soluble and insoluble hemicellulose contents of raw barley. Present, and other, evidence has shown the superior malting properties of Proctor and it is suggested that this may be related, in part, to the comparative figures for the hemicellulose contents of the barleys and their malts.

SECTION 3.

The Purification of the β-Glucosanase System and the Enzymolysis of β-Glucosan.

INTRODUCTION.

Advancement of our knowledge of the enzyme degradation of cell-wall materials has been hampered by a combination of factors. In the first place, unmixed substrates were not available until recently, when a great advance was made by isolation of a barley β-glucosan by Preece and Mackenzie (1952), and secondly, methods for the purification and separation of enzymes or enzyme systems must still be regarded as being rather inadequate. However, it is generally recognised that, for distinction of different enzymes in a mixture, recourse may be had to several devices, including the use of a specific substrate, the use of different means (such as viscosity drop, reducing power increase) to measure different aspects of activity, and the use of specific inactivators. In work on the present topic, a combination of these devices has proved necessary.

Lüers and Volkämer (1928) can be credited with the first attempt to purify enzymes capable of hydrolysing non-starchy barley polysaccharides. It was claimed that the green-malt enzyme or enzymes concerned in this hydrolysis could be precipitated from solution by alcohol without loss of activity, whilst a partial purification was achieved by adsorption on alumina.

In their investigations of the enzymolysis of mixed polysaccharides resembling the amylans of O'Sullivan (1882), but later described as barley gums, Preece and Ashworth (1950) obtained enzyme preparations of enhanced cytolytic activity by extraction of green malt with an appropriate buffer solution and subsequently fractionating /

fractionating the extract with alcohol. The activity values were determined by measuring the increase of reducing power of gum-enzyme mixtures at pH 5.0 and 37°C. The most active fraction (yield × activity) was obtained at an alcohol concentration of 65%. It is worthy of note that highly active enzyme preparations were obtained without fractionation when the malt was extracted with 20% alcohol and the enzymes were precipitated by increasing the alcohol concentration to 79%. The "cytolytic" factor, however, was not found at that time in raw barley and was thought to be first formed only after steeping and growth of the barley corn.

Scandinavian workers have employed artificial substrates, water-soluble derivatives of cellulose, in investigations of this nature. For instance, Kristiansson (1950), using hydroxyethyl-cellulose, demonstrated that barley malt "cellulase" loses two-thirds of its activity on heating at 60°C. and pH 5.0 for 15 minutes. On the other hand, Sandegren and Enebo (1952) have described a viscometric method for the determination of "cellulase" activities of aqueous extracts of barley or malt. However, it is clear that the results obtained by the use of an artificial substrate do not accurately parallel those which can be achieved using β-glucosan as substrate (vide infra). Sandegren and Enebo found the pH optimum of unpurified "cellulase" from barley and malt to be 5.0 while the barley preparation exhibited a low activity at 70°C.

In a further investigation Enebo, Sandegren and Ljungdahl (1953) devised a method which achieves a considerable purification of the "cellulase" system. The enzymes were first precipitated from an aqueous extract by ammonium sulphate between the limits of 45% and 75% concentration of the salt. This crude enzyme preparation was redissolved in water and the solution dialysed to remove ammonium /

ammonium sulphate. The enzymes were reprecipitated from the clear dialysate by acetone between the limits of 35% and 75% concentration by volume. The final product was dried with acetone and ether and was about 40 times as active as the original green-malt preparation on a nitrogen basis. This method has since been widely applied to this particular problem. These workers also studied the inhibitory effects of cellobiose and lactose on the "cellulase" activities of crude, green-malt extracts and purified, green-malt preparations respectively. At the same time, they noticed that the activities of the purified preparations, unlike those of the crude, green-malt extracts, were stimulated by the addition of glucose, mannose or xylose. The mechanism of activation of purified, green-malt "cellulase" by glucose they explained by the assumption of at least two enzymes in the system, a typical hydrolytic β-polyglycosidase and a non-hydrolytic "transglycosidase" which, for its action, is dependent upon the presence of such monosaccharides. In agreement with this and using a rye araboxylan, Preece and Hobkirk (1955) gave reason to suppose that wheat enzymes could effect transarabinosylation.

Under the action of barley enzymes, β-glucosan in solution undergoes a rapid fall in viscosity; using a modification of the method proposed earlier by Sandegren et al. (loc. cit.), this fall was made the basis for the assessment of cytoclastic action, in respect of β-glucosan, by Preece and Aitken (1953). On the assumption that rapid viscosity diminution accompanied by relatively small liberation of reducing groups was caused by attack on molecular chains at points remote from the ends, and with the realisation that β-glucosan is related to lichenin rather than to cellulose, Preece, Aitken and Dick (1954) preferred the name endo-β-glucosanase /

β-glucosanase for the enzyme or enzyme system responsible rather than "cellulase". These workers were also seeking means of purifying barley enzymes, and an important feature in their purification procedure is that, prior to precipitation of the enzymes, appreciable autolysis was allowed to occur by allowing the extracts to stand overnight at room temperature, thus reducing contamination by hydrolysis products to negligible proportions. This precaution followed by addition of ammonium sulphate in 40% concentration was the most effective in yielding preparations of enhanced activity.

However, when barley enzymes act on β-glucosan (25°C., pH 5.0), the rapid diminution in solution viscosity is accompanied by a rapid production of cellobiose and glucose. It can be shown that a cellobiase is abundantly present in raw barley, so that with cellobiose being undoubtedly formed, the presence of glucose is easily accounted for. The formation of cellobiose, however, unaccompanied by other oligosaccharides cannot be accounted for by endo-attack alone, and it appeared probable that the disaccharide was being liberated from chain ends by an exo-β-glucosanase acting in a manner analogous to the attack on amylose by β-amylase. Moreover, on the assumption that endo-attack, at least in the earliest stages, would produce one additional reducing group each time a chain was split (thus decreasing the average molecular size and the viscosity to one-half), it could be shown that more reducing groups were produced by barley enzymes than could be accounted for by such action alone. The additional reducing groups must, on this assumption, have arisen from cellobiose production and from the glucose formed from this by cellobiase action (and perhaps by hydrolysis from unobserved laminaribiose); determination of these additional reducing groups would therefore give, as a first approximation /

approximation, an estimate of exo-β-glucosanase activity, augmented naturally by the concomitant and dependent cellobiase activity. The concept that to halve the viscosity by endo-β-glucosanase action involves doubling the reducing power of the substrate was of considerable value in subsequent investigations, and its further application and limitations are discussed in more detail below.

Bass, Meredith and Anderson (1953), having worked with mixed barley gums, and with enzyme preparations from barley and green malt, stated that the presence of the viscosity-decreasing factor in their barley preparations was undoubted, but it was not clear whether barley yielded the exo-enzyme, whilst Bass and Meredith (1955) subsequently reported the action of enzyme preparations from green malt on β-glucosan. Chromatographic analyses revealed the early production of glucose followed after some thirty hours by a trace of cellobiose and later by a second disaccharide, possibly laminaribiose. Meantime a high molecular oligosaccharide had appeared near the starting line of the chromatogram, to be followed later by apparently three oligosaccharides of greater chromatographic mobility and, presumably, of smaller molecular size. From a consideration of the rates of appearance of the individual products, it was again concluded that the combined actions of endo- and exo-enzymes provided the explanation most consistent with the facts, the two enzymes being now described as endo-β-polyglucosidase and exo-β-polyglucosidase.

The danger in relating the results obtained in experiments employing artificial substrates to what happens in the intact grain has been emphasised by various workers, notably Thomas (1956), who was investigating the "cellulases" obtained from Stachybotrys atra. It /

It was shown that no cellobiase was present but the preparations did attack cellotriose and cellotetraose. Thomas also examined the effect of various compounds on "cellulase" activity, when he observed total inhibition by mercuric acetate and partial inhibition by cupric sulphate, lead acetate and potassium cyanide. The failure of iodoacetic acid, a thiol-specific reagent, to inhibit "cellulase" activity could be explained from a qualitative examination of the amino acids present in such preparations from Stachybotrys atra in which no sulphur-containing acids, such as cysteine and methionine, could be detected. In this investigation there was no inhibitory effect on adding sugars at a concentration of 0.2% which is not contrary to the work of Enebo et al. (loc. cit.) who found that cellobiose and lactose in concentrations lower than 1% had a stimulating effect but in concentrations above 1% the activity was decreased.

In recent years Conchie et al. (1954, 1955) have been investigating the selective inhibition of glycosidases by the corresponding aldonolactones. They observed powerful and selective inhibition of β-glycosidase, for example cellobiase, by glucono -1:4- lactone and to a lesser extent by glucono -1:5- lactone. The disadvantage is that, as a result of the mutarotation of the lactones to gluconic acid, which has no inhibitory effect, of the glucono -1:4- lactone originally present after 119 hours at room temperature 59% remained whilst only 18% of the glucono -1:5- lactone remained unchanged.

Recently Aitken et al. (1956) presented evidence regarding the manner in which the β-1,4- and β-1,3- linkages are distributed within the β-glucosan chain. In their investigations these workers studied the breakdown of insoluble cellulose preparations, soluble /

soluble derivatives of cellulose, laminarin and β-glucosan by unheated and heated culture filtrates of the fungus Myrothecium verucaria. They concluded that the only explanation of their results was a grouping of the β-1,3- and β-1,4- linkages in the β-glucosan molecule. Work of a similar nature has been carried out by the Birmingham school in connection with an α-glucosan isolated from Aspergillus niger. Barker, Bourne and Stacey (1952) described the isolation of this polyglucosan which, when subjected to partial hydrolysis, yielded, among other products, a trisaccharide fraction. When this fraction was examined chromatographically by the benzylamine method of Bayly and Bourne (1953) it appeared to be homogeneous, but from the R_F value the presence of both a -1,3- and -1,4- link was inferred. However, Foster (1953) had shown that migration of neutral sugar derivatives, in ionophoresis, occurs at an alkaline pH in the presence of borate ions when weakly charged complexes are formed. Therefore, Barker et al. (1953) in collaboration with Foster (loc. cit.) examined the trisaccharide fraction ionophoretically, when the presence of two components was shown. This result was further evidence in support of the structure of alternate α-1,3- and α-1,4- linkages postulated by Bayly and Bourne (loc. cit.), since on partial hydrolysis such a structure would afford a mixture of trisaccharides linked 1,3- 1,4- and 1,4- 1,3-.

To sum up, degradation of water-soluble β-glucosan by barley enzymes appears from the above evidence to involve (a) an endo-β-glucosanase system; (b) an exo-β-glucosanase; (c) cellobiase; and (d) possibly a system permitting transglucosylation, though whether this last system, if it exists, is independent of the others or inherent in their actions remains to be determined. The purpose of /

of this section is to provide further evidence in support of this view of the complexity of the degradative scheme, to describe methods for obtaining enzyme preparations of enhanced purity and to examine the enzymolysis products obtained from β-glucosan when β-glucosan is subjected to attack by enzyme systems possessing varying amounts of exo-β-glucosanase.

EXPERIMENTAL.

Preparation of β-glucosan.

In general, the method of Preece and Mackenzie (1952) has been followed with some modifications. It is emphasised that the barley must be ground in an ordinary coffee-mill, since the use of a hammer-mill (approximately 3,000 revolutions per minute) has been shown, in the present work, to give rise to extensive degradation of the β-glucosan, i.e. the preparation has no appreciable viscosity. Furthermore, later preparations of extremely high viscosity have been obtained after submitting the ground grain to $4 \times \frac{1}{2}$ hr. treatments with boiling 80% (v/v) ethanol, a further precaution against the existence of residual enzymic activity being taken by the addition of a suitable volume of mercuric chloride solution to the water extracts (cf. Lindet (1903)). The β-glucosan obtained in this way is quite stable in solution.

Extraction and precipitation of barley enzymes.

300 gm. of barley were extracted with 1,000 ml. of 0.6% (w/v) sodium chloride solution, with constant stirring, for 1 hr. at room temperature. The extract was filtered water-bright and allowed to autolyse overnight at room temperature. This autolysis period (approximately 24 hr.) is an important step in the purification of the enzyme preparations. Following Preece, Aitken and Dick (1954), who found this to be a most effective means of purifying /

ing the β-glucosanase system, ammonium sulphate was added in graded amounts and all precipitates obtained below 40% (w/v) concentration of the salt were discarded. The precipitate obtained at the 40% (w/v) level was centrifuged down and the concentration of ammonium sulphate in the supernatant liquor increased to 50% (w/v). These two fractions were dissolved separately in small volumes of distilled water and each solution was re-fractionated at the respective salt concentrations. The precipitates were re-dissolved in distilled water and the solutions were dialysed for 3 days against running water, after which the enzymes were precipitated by adding slowly four volumes of acetone with stirring. Drying was effected by increasing the strength of the acetone gradually and was completed by storing *in vacuo*.

Activity determinations by the method of Preece *et al.* (*loc. cit.*) showed that the 40 and 50% (w/v) fractions possessed endo-β-glucosanase activities of 0.0417 and 0.0622 units (reciprocal specific viscosity increase per hr. per mg. of enzyme preparation) respectively. Therefore, in future, unless stated otherwise, the activity values given will refer to those preparations obtained at 50% (w/v) ammonium sulphate concentration after removal of material which settles out at the 30% level, *i.e.* a combination of the 40 and 50% fractions will be employed.

Influence of pH on extraction.

In this investigation the same sample of barley was employed in order to provide a comparison with the above method of extraction; extract concentration in all instances was 30% (w/v). The ground grain was extracted for 1 hr. with constant stirring with buffer solutions of varying pH values, prepared according to Britton (1942). The extractants in these experiments were McIlvaine's /

McIlvaine's citrate-phosphate buffer (pH 2.2-8.0) and the Universal Buffer of Prideaux and Ward (pH 4.0-11.0), and so a certain degree of overlap was attained. Extraction completed, the extracts were filtered water-bright and they were allowed to autolyse for approximately 24 hr., after which the pH values of the extracts were determined electrometrically. This was followed by dialysis (3 days) of the extracts to remove salts and the products of autolysis. Each dialysate was then treated with ammonium sulphate to precipitate the 30% (w/v) fraction, which was centrifuged off and discarded. The concentration of ammonium sulphate was increased then to 50%, the resulting precipitate being retained after centrifugation. Each precipitate was dissolved in 25 ml. of distilled water and dialysed for 3 days. Recovery and drying of the preparations were carried out as above.

In the extraction series employing Universal Buffer the yields of enzyme increased as the pH of the buffer increased, this possibly being the result of the extraction of greater amounts of hemicellulosic materials as the hydroxyl ion concentration increases. A further point of interest is that initial buffer pH values below 6.0 are increased during extraction, whilst those above 6.0 are decreased.

Table VII shows the results of activity determinations by the methods of Preece et al. (loc. cit.) and it will be noticed that there is a broad zone of optimal activity in the range 4.2-5.1. From these results there can be no doubt of the greater efficiency of buffer extraction, especially by the citrate-phosphate buffer, in yielding preparations of enhanced activity because all determinations have employed the same sample of barley.

Figure 14 illustrates the effect of pH upon the endo- and exo-β-glucosanase /

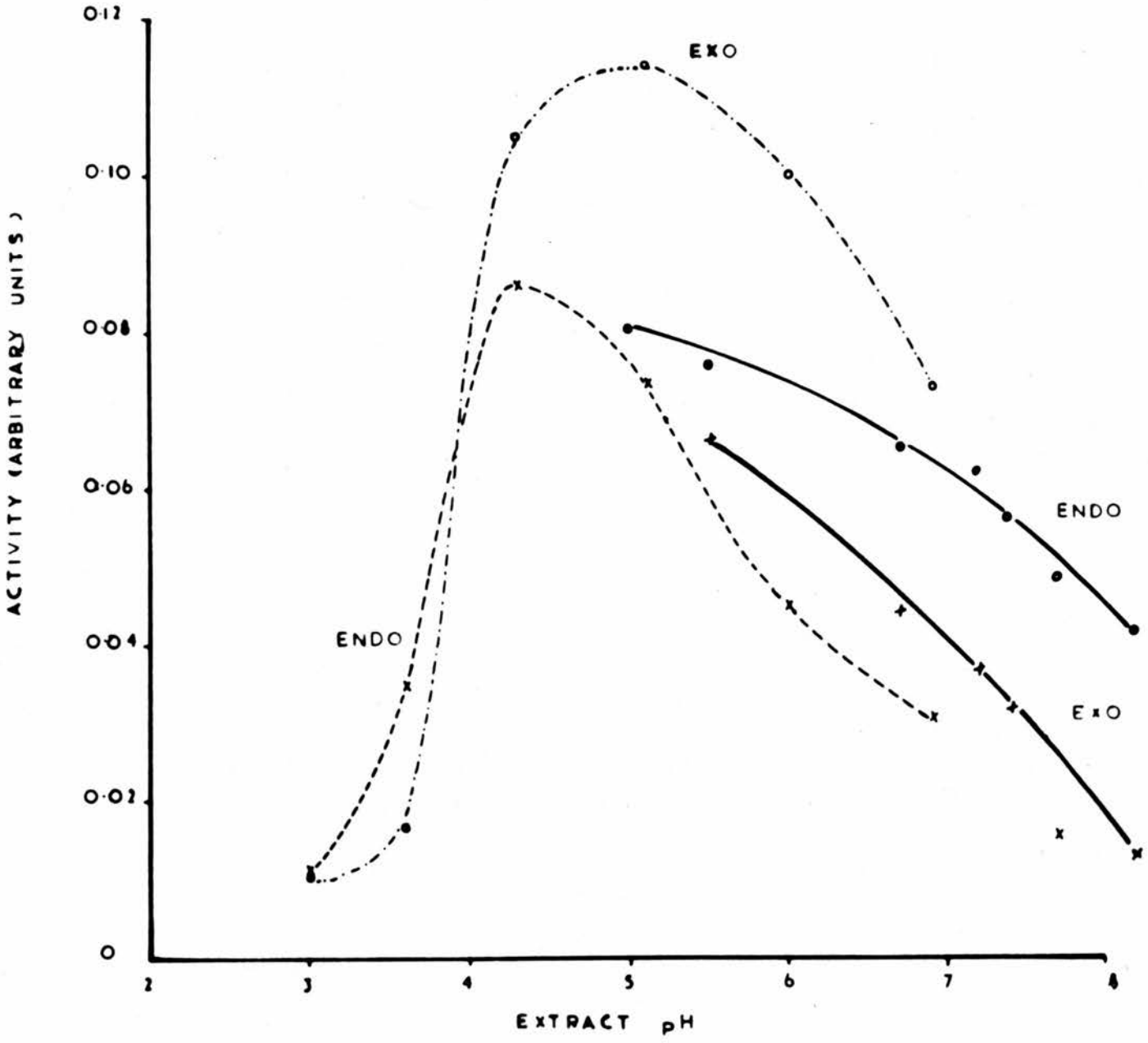


Figure 14.

Influence of extract pH on β-glucosanase activities of enzyme preparations from barley. Broken lines, citrate-phosphate buffer; full lines, Universal buffer.

β-glucosanase activities.

Influence of pH on precipitation.

600 gm. of the same sample of barley were extracted with 2,000 ml. of citrate-phosphate buffer of pH 4.2, as above, and after autolysis the extract (pH 4.4) was dialysed for 3 days. The dialysate had a pH value of 6.4, owing to the removal of salts, and it was divided into 8 × 125 ml. aliquots. These were then adjusted separately to the required pH values from 4.0-5.2 using normal acetic acid which was added drop by drop with rapid stirring to eliminate local pH effects. This done, each solution was treated with ammonium sulphate to 30% (w/v) concentration and the resulting precipitates were discarded. As a result of the acidity of the salt a change in pH occurred and, therefore, it was necessary to re-adjust to the original values with normal acetic acid for preparations precipitated between pH 4.0-5.0 and with normal ammonia solution for those above this range. The ammonium sulphate concentration was adjusted then to 50% (w/v) concentration, the resulting precipitates, after centrifugation, being dissolved in 25 ml. of distilled water, dialysed for 3 days, and finally precipitated and taken to dryness with acetone in the usual way.

The results of viscometric, reductiometric and cellobiase determinations are shown in Table VIII, where it is observed that the optimum pH for precipitation is 4.4, but no separation of the three activities was effected. Figure 15 shows the influence of pH on the precipitation of the β-glucosanase system.

Method adopted.

From these results it is seen that, within the same sample of barley, a combination of extraction and precipitation techniques at the respective optimum pH values has led to an increased purification

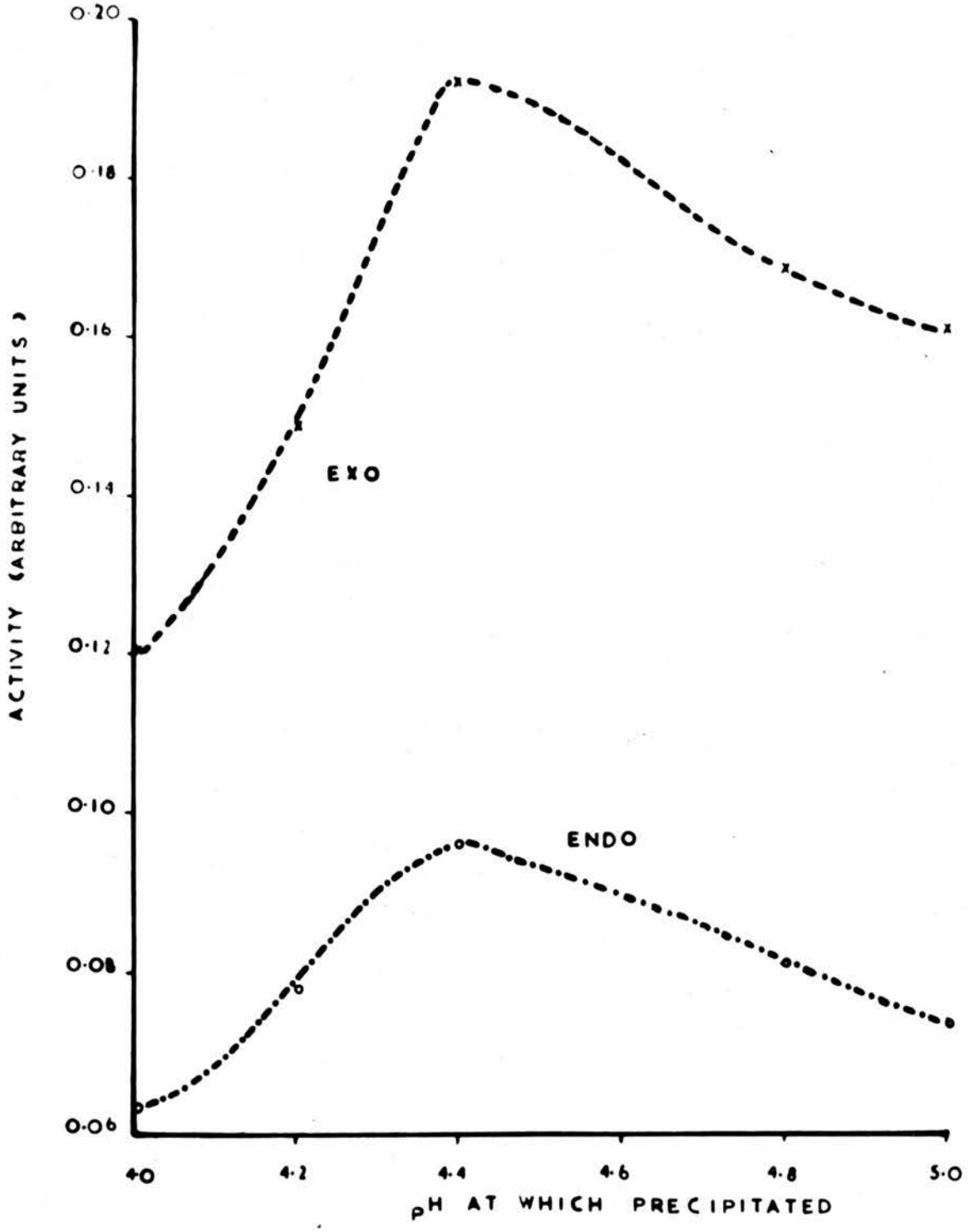


Figure 15.

Influence of pH on precipitation of β-glucosanase.

of the β-glucosanase system of the barley grain.

It was therefore decided to apply this method to a survey of the enzyme potentialities of the five raw cereals, with the results shown in Table IX. 300 gm. of the ground cereal were extracted under standard conditions with 1,000 ml. of citrate-phosphate buffer solution of pH 4.5, giving extract pH 4.6-4.7 after allowing to autolyse overnight. The extract pH was adjusted to 4.5 with normal acetic acid and the extract was dialysed for 2-3 days. After re-adjustment to pH 4.5 material of low activity was precipitated by the addition of 30 gm. of ammonium sulphate per 100 ml. of extract, this fraction being discarded. Once more adjusting the pH value to 4.5 by means of normal acetic acid, an additional 20 gm. of salt per 100 ml. of original extract was added, the resulting precipitate, after centrifugation, being dissolved in a small volume of distilled water and the solution dialysed for 2 days. The enzyme preparation was finally precipitated with acetone and taken to dryness in the usual way. Thymol was used as antiseptic throughout the preparations. The results of Table IX show endo-β-glucosanase activities 10-20 times as great as those reported by Preece et al. (loc. cit.) for crude, acetone-precipitated preparations from the corresponding cereals; the figures for total exo-activity have increased, however, in much greater proportion, except in the case of barley.

Table IX additionally shows a numerical estimate of cellobiase activity, determined and calculated as before. The enormous cellobiase activity of the barley preparation, and the relatively insignificant figures for rye and maize, will be noted. Plate IV provides evidence in support of these values for the cellobiase activities of the cereals, production of glucose from cellobiose

by /

PLATE IV.

Chromatogram to show the variations in cellobiase activities of the five common cereals.

(Solvent, butanol: acetic acid: water).

C. - control.

O. - oats enzyme preparation.

M. - maize enzyme preparation.

B. - barley enzyme preparation.

W. - wheat enzyme preparation.

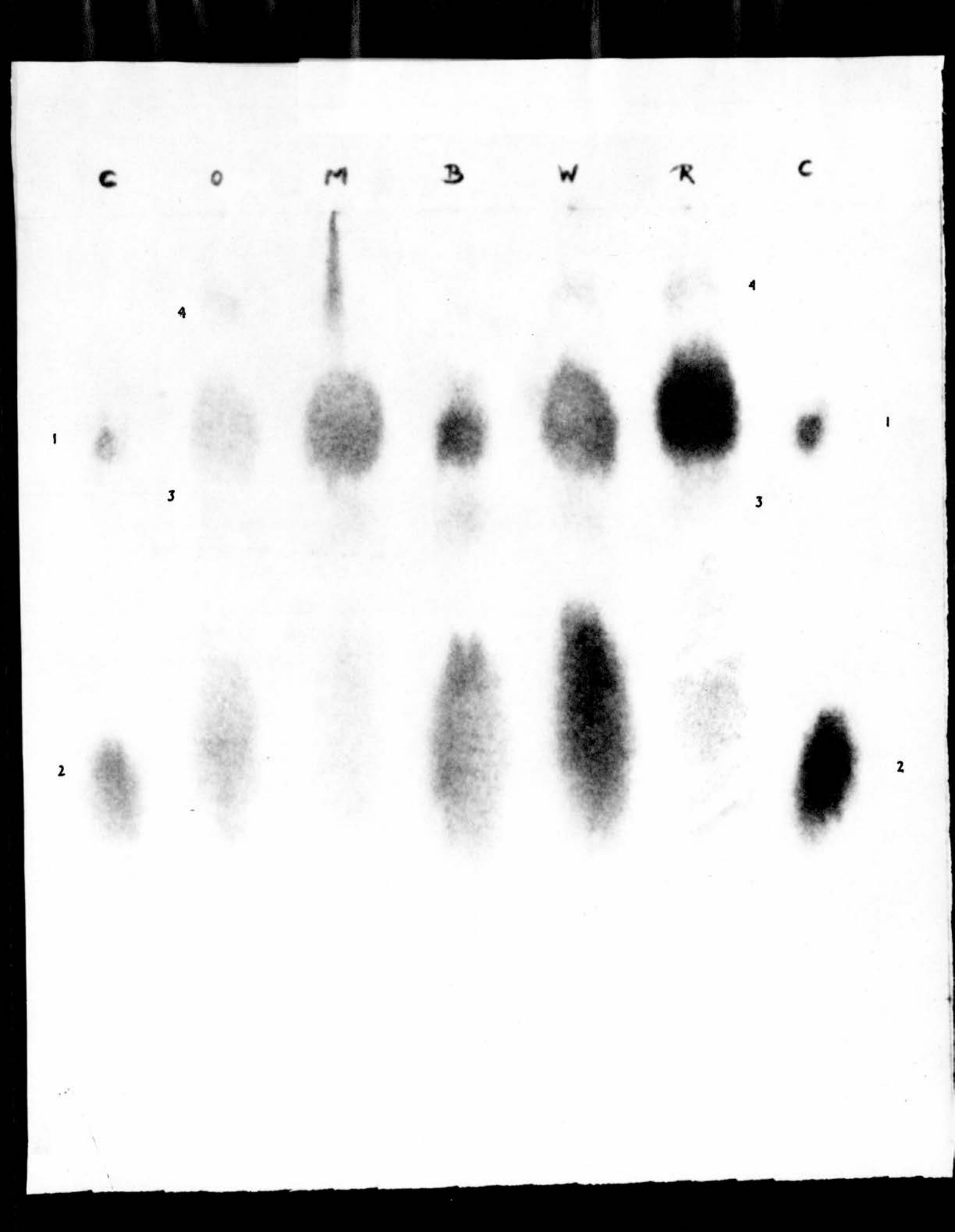
R. - rye enzyme preparation.

1. - cellobiose.

2. - glucose.

3. - laminaribiose?

4. - gentiobiose?


C
O
M
B
W
R
C
4
4
1
1
3
3
2
2

by the maize and rye preparations being extremely small.

The crude, acetone-precipitated green-malt and finished malt preparations obtained, as described earlier, during the malting of Ymer barley were independently re-dissolved in water and submitted to ammonium sulphate fractionation. The 30% (w/v) fraction was discarded, but the fraction obtained at 50% (w/v) concentration was dissolved in distilled water and re-fractionated to remove any residual 30% material. The final precipitate was re-dissolved in a small volume of distilled water, dialysed for 2 days, precipitated with acetone and taken to dryness. The results of activity determinations, shown in Table IX, are of interest when compared with the activities of the other raw cereals. The endo-β-glucosanase activity of the finished malt preparation is approximately 230 times as great as the activity of the crude Ymer raw barley preparation shown in Table II, whilst ammonium sulphate fractionation has effected an approximately 100% increase in the activity of the Ymer finished malt preparation.

Differential inactivation.

Reference to Figure 14 will show that, with citrate-phosphate buffer and from pH 4.0 upwards, the figure for arbitrary units of exo-activity always exceeds that for endo-activity; with the Universal Buffer (phosphoric acid - boric acid - phenylacetic acid) and above pH 5.5 the reverse is the case, suggesting a degree of inactivation of the exo-enzyme system by either boric or phenylacetic acid.

It had been shown already (Table IX) that maize possesses a low exo-β-glucosanase activity and, therefore, preliminary experiments were begun with preparations from this source. The fraction obtained at an ammonium sulphate concentration of 50% (w/v), after discarding /

discarding the 30% fraction, was dissolved in 75 ml. of distilled water and this solution was divided into 3 × 25 ml. aliquots which were treated separately, as follows:

(A) Dialysed for 3 days.

(B) Phenylacetic acid (5 mg. per ml.) was dissolved in the solution which was dialysed for 3 days. A precipitate, formed on the addition of the reagent, was removed by centrifugation.

(C) Borax (5 mg. per ml.) was dissolved in the solution, which was also dialysed for 3 days.

The results of activity determinations are shown in Table X, where it is observed that a degree of inactivation is effected by both reagents and, therefore, it was decided to examine their effects on a system possessing a greater exo-activity, e.g. the barley β-glucosanase system, and also under conditions which allow prolonged contact with the borate and phenylacetate ions. In these experiments the general method of working was as follows: A mixture of barley enzymes, prepared by the method outlined above, was dissolved in an appropriate volume of water, treated with the specified amount of reagent under test, and maintained at the desired temperature for the stated interval of time. The solution was then dialysed at room temperature for 3 days, except where stated, and the enzymes were recovered with acetone and thereafter taken to dryness. It will be observed that a different enzyme preparation was employed in each series of experiments, and each control represents part of the enzyme preparation which had been dissolved in water, dialysed for 3 days, and thereafter recovered; furthermore, throughout each series, a fixed sample of β-glucosan was employed in the determination of endo- and exo-β-glucosanase activities /

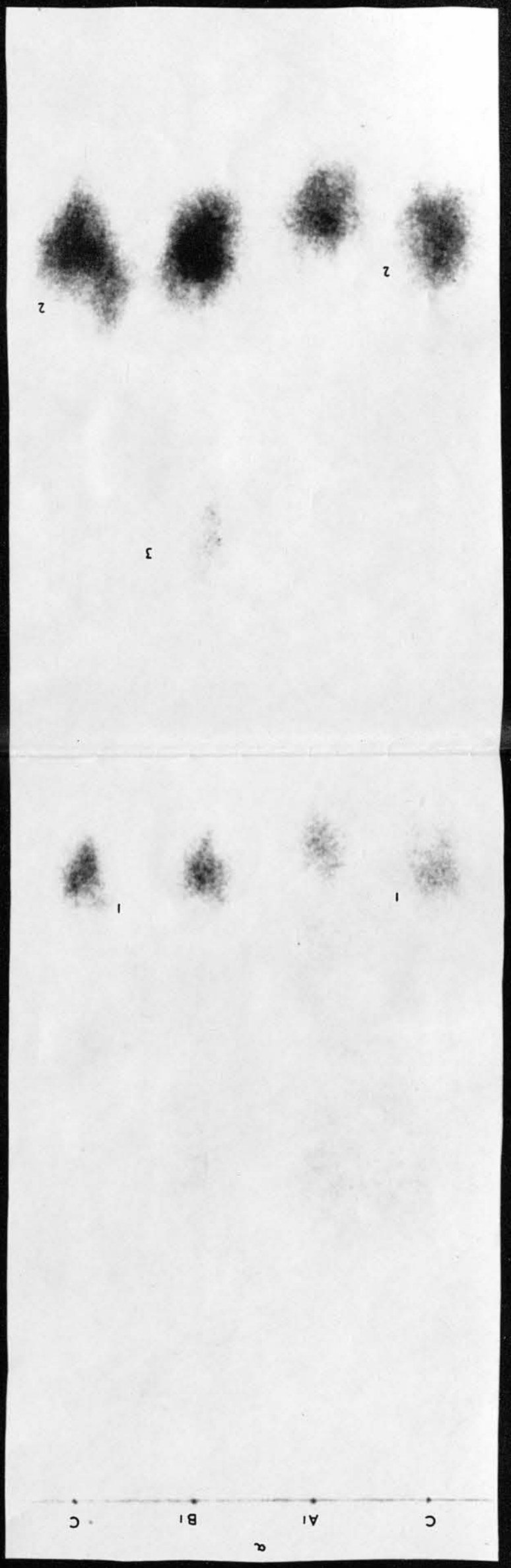
activities. The individual effects of borax and phenylacetic acid on the three aspects of activity are shown in Tables XI and XII respectively.

From these results the differential effect of the phenylacetic acid is very clear and leaves no doubt as to the mixed nature of the enzyme preparation (Figure 16). It was observed that the addition of phenylacetic acid resulted in the formation of a precipitate which increased in amount with the concentration of reagent employed. Although the nature of this precipitate was not examined, it is possible that it consists largely of the inactivated protein.

Further experiments were carried out with a view to effecting selective inactivation of the endo-β-glucosanase and cellobiase systems, the results of activity determinations being shown in Table XIII. Heat treatments are destructive of both endo- and exo-activities, though the exo-activity - confirming what is shown in Figure 14 - is also the more susceptible to destruction by hydrogen ions. The most successful treatment, in the sense that it permits recovery of nearly 90% of the original endo-activity, but unaccompanied by exo-activity, is that with phenylmercuric nitrate. Cellobiase activity is not shown here; in fact, no method has yet been found for destroying cellobiase activity whilst retaining any substantial amount of endo-activity.

Selective inhibition of cellobiase activity by glucono -1:4- lactone was attempted, according to Conchie et al. (1954, 1955). 4 ml. of a 0.6875% (w/v) solution of cellobiose were mixed with 1 ml. of acetate buffer (pH 5.0) and the temperature was raised to 25°C. To this solution 4 ml. of glucono -1:4- lactone solution (5 mg. per ml.), already at 25°C., were added. The solutions were mixed /

b



a

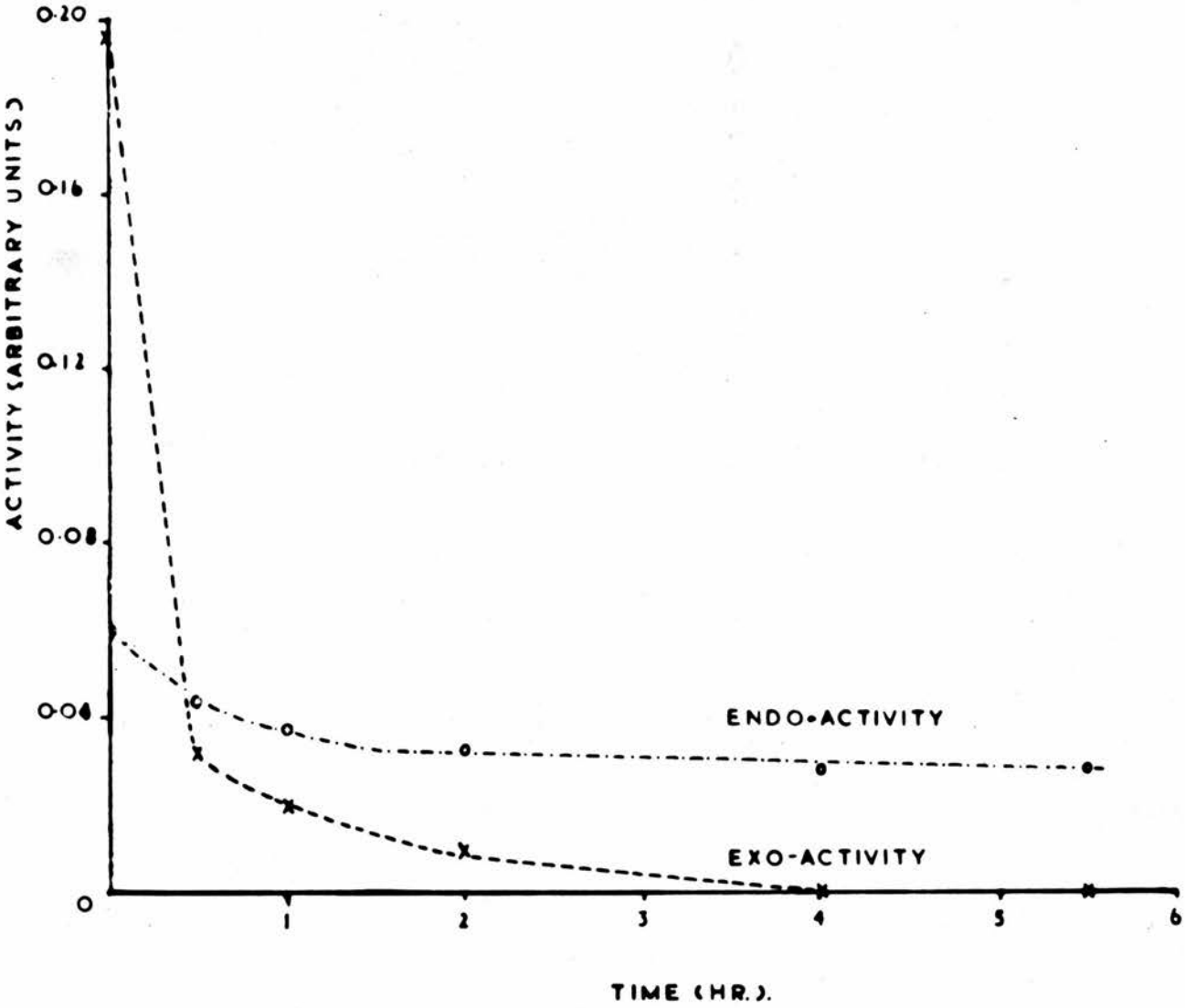


Figure 16.

Influence of phenylacetic acid (5 mg. per ml.) on β-glucosanase activities of enzyme preparations from barley.

PLATE V.

Chromatogram to show the effect of glucono-1:4-lactone on cellobiase activity of a raw barley enzyme preparation.

(Solvent, butanol: acetic acid: water).

C. - control.

A1. - reaction of enzyme with cellobiose.

B1. - reaction of enzyme with cellobiose plus glucono-1:4-lactone.

A2. - cellobiose control.

B2. - glucono-1:4-lactone control.

1. - cellobiose.

2. - glucose.

3. - glucono-1:4-lactone.

mixed by stirring and 2 ml. of a solution of a barley enzyme preparation, which had also been maintained at 25°C., were added immediately. Suitable controls were run concurrently, the reactions being terminated after 1 hr. by heating at boiling point in a water-bath for five minutes. The results, shown in Plate V, indicate that any inhibition of cellobiase activity by glucono -1:4-lactone must be small.

Following work by Meredith et al. (1953, 1955) it was decided to study the effect of papain on the barley β-glucosanase system. A sample of barley was extracted (150 gm. in 500 ml. of 0.6% sodium chloride solution), allowed to autolyse overnight and dialysed for 3 days. To the dialysate was added papain to 0.025% concentration and after allowing to stand at room temperature for 4 hr., the solution was treated with four volumes of acetone and the precipitate was taken to dryness. A control was run also and the results of activity determinations are shown in Table XIII. It was shown that the sample of papain had no effect upon the viscosity of the β-glucosan solution.

Chromatographic investigations.

Direct chromatography of β-glucosanase reaction mixtures was impracticable, owing to the complex nature of the products obtained in most cases. The technique adopted was to allow the selected enzyme preparation to act on 0.5% β-glucosan solution (25°C., pH 5.0) until the specific viscosity had reached a steady value (usually in the neighbourhood of 0.10); the time taken, of the order of 20-30 hr., and the actual value reached, depend on the enzyme concentration employed, as shown in Figure 17. The conversion liquor was then treated with four volumes of 95% (v/v) ethanol when high-molecular material was precipitated. This precipitated material /

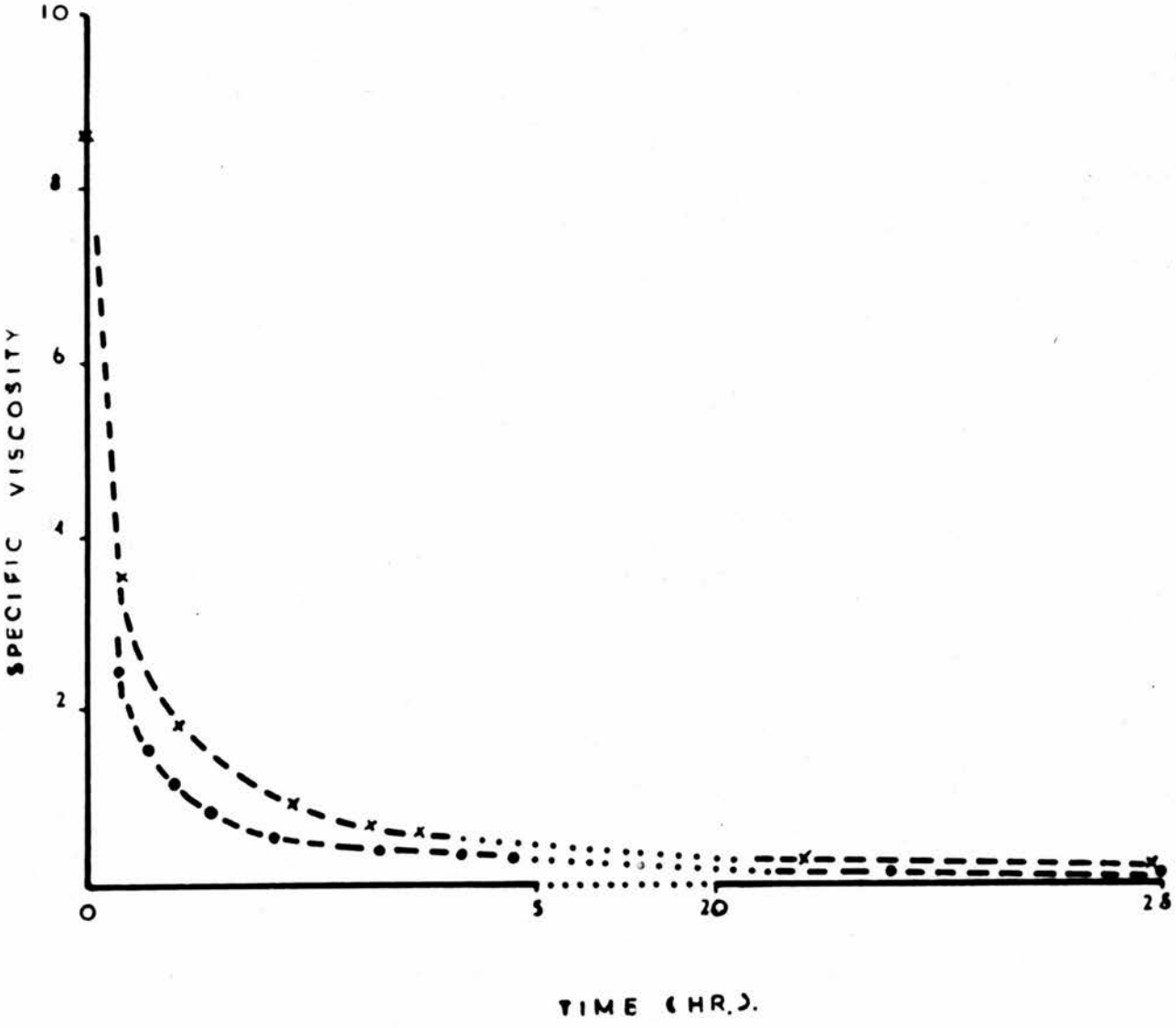


Figure 17.

Approach of specific viscosity of β-glucosan solutions to zero under the action of endo-β-glucosanase. One curve (x) represents one-half the enzyme concentration of the other (o).

material is of some interest and will be referred to as β-glucosan "dextrin". The mother liquor, after concentration to small volume, was applied to a charcoal-celite column (vide infra).

Determination of molecular size of β-glucosan "dextrins".

The dextrins produced in those conversions run to the limit of viscosity diminution are of interest in that enzymolysis beyond this point appears to be extremely slow (Figure 17) and, therefore, it was decided to determine their molecular size.

In the first instance the molecular size of a sample of a dextrin was determined by iodimetry, the sample having been obtained by the action on β-glucosan of a barley enzyme which had been treated with phenylacetic acid to inactivate exo-β-glucosanase. The chain-length of the polysaccharide, after standardisation of the method with glucose, was found to be of the order of 18 glucose units. However, the accuracy of the iodimetric method is suspect beyond 7-8 units.

A colorimetric method put forward by Nelson (1944) was employed in subsequent determinations of molecular size. This method employs an arsenomolybdate colour reagent and a modified Somogyi copper reagent, whilst standardisation against the fundamental unit of the polysaccharide is necessary. Standardisation presented a difficulty because of the presence in the β-glucosan molecule of β-1,3- and β-1,4- linkages in equal numbers as stated by Aspinall and Telfer (1954). However, the method was standardised against laminaribiose and cellobiose (glucose is not regarded as the fundamental unit), the limit of concentration of these being fixed at 0.6×10^{-6} gm. mole. The sugar solution (2 ml.) was placed in a 25-ml. volumetric flask and in a boiling water-bath for ½ hr. with the copper reagent (2 ml.). After cooling /

cooling in cold water for $2\frac{1}{2}$ minutes the arsenomolybdate solution (2 ml.) was added with shaking; after a further minute water was added to 25 ml. The intensity of the colour developed was measured $\frac{1}{2}$ hr. after dilution against that developed in a control from which sugar was absent. The molecular size of the polysaccharide could then be obtained by comparing its reducing power with that of laminaribiose and cellobiose. That glucose is not the lowest member of the homologous series can be demonstrated by the small error obtained by comparing the reducing power of the monosaccharide with that of a disaccharide. However, it was found that laminaribiose and cellobiose gave two distinctly different standardisation graphs, which is in agreement with the fact that a certain concentration of laminaribiose always gives a lower reducing value than the same concentration of cellobiose. The chain-length of the dextrin when referred to laminaribiose is 16 units, and 28 glucose units when the fundamental unit is taken to be cellobiose. In view of the evidence of Aspinall and Telfer (loc. cit.) it seems quite reasonable to take the mean value, 22 glucose units, as representing the degree of polymerisation of the limit dextrin. This result is of the same order as that obtained by iodimetry, the barley enzyme and β-glucosan preparations employed being different in each instance.

In conclusion, therefore, the molecular size of the dextrins resulting from these experiments run to the limit of viscosity diminution appears to be of the order of 20 glucose units, slight variations occurring according to the barley enzyme concentration employed.

Fractionation /

Fractionation of sugars.

Charcoal-celite columns were prepared by mixing equal quantities of acid-washed charcoal and Celite 545 with water (325 ml. per 100 gm. of mixture). The slurry was poured into a glass column, 2-3 inches at a time, which contained a piece of glass wool as a support. After allowing the columns to settle they were washed with approximately 500 ml. of water. The mother liquors, concentrated to small volume, were then applied to the columns and fractionation of the sugars was effected by the method of Whelan et al. (1953), which involves extracting the columns successively with (a) water, (b) 7.5% (v/v) ethanol and (c) 30% (v/v) ethanol. The water-eluates and the 7.5% ethanol solutions, after appropriate concentration, were submitted to paper chromatography in the ordinary way, employing the upper layer of the system butanol:acetic acid:water (40:10:50) as the developer. The 30% ethanol eluates, after concentration, were chromatographed according to Bayly and Bourne (1953). To one spot of the test solution, on the starting line, is added one spot of a 10% (w/v) solution of benzylamine in methanol and the paper is heated at 85°C. for five minutes to effect the formation of the benzylamine derivatives. These workers have stated that it is inadvisable to apply more than one spot of the test solution, but in the present work where the concentration of the oligosaccharides was quite small no adverse effects have been observed by applying up to ten spots. Development was by the system butanol:ethanol:ammonia: water (40:12:1:20). The oligosaccharide derivatives were detected, after 24-36 hr., by spraying with a 0.25% (w/v) solution of ninhydrin in ethanol, the colour developing at room temperature after 1 hr. Maximum colour intensity was attained only after approximately /

approximately 2 days.

For purposes of comparison, five purified enzyme preparations were selected at random from those available to show the behaviour towards β-glucosan of enzymes from raw barley, raw barley enzymes treated with phenylacetic acid to inactivate exo-β-glucosanase, green-malt enzymes, enzymes of finished malt, and enzymes from maize. All reaction mixtures were comparable with respect to endo-β-glucosanase activity, and, from what has been shown above, it will be realised that exo-activity will be greatest in the reaction mixture employing raw barley enzymes, less in the reaction with maize enzymes and still less in that reaction employing the green-malt preparation, whilst exo-activity is absent in the reactions with finished malt and the inactivated barley preparations. The enzyme concentrations, in all instances, were chosen in such a way that reduction of specific viscosity to one-half occurred at 1 hr. (initial specific viscosity of substrate = 18.10) and aliquots were withdrawn at 2, 6 and 29 hr., these being treated in the appropriate manner (the limit of viscosity diminution occurred at a specific viscosity of 0.33). Chromatographic analyses failed to detect glucose and disaccharide production after 2 hr. in all but the reaction mixture employing the raw barley enzyme preparation, and only then these were detected with very great difficulty. Glucose production at 6 and 29 hr. revealed a similar pattern, which is shown in Plate VI, the raw barley enzyme producing the greatest amount whilst the maize and green-malt preparations yielded, in comparison, only negligible amounts. The monosaccharide was absent in those reaction mixtures employing finished malt and inactivated barley preparations. Plate VII shows the common pattern of disaccharide production after 6 and 29 hr. Cellobiose and /

PLATE VI.

Chromatogram to show the production of glucose (eluted from column by water) by the action on β-glucosan of enzyme preparations from different sources. (at 6 and 29 hr.).

(Solvent, butanol: acetic acid: water).

C. - control.

A1. - reaction with green-malt preparation.

A2. - reaction with finished malt preparation.

A3. - reaction with maize preparation.

A4. - reaction with raw barley preparation.

A5. - reaction with inactivated barley preparation.

1. - glucose.

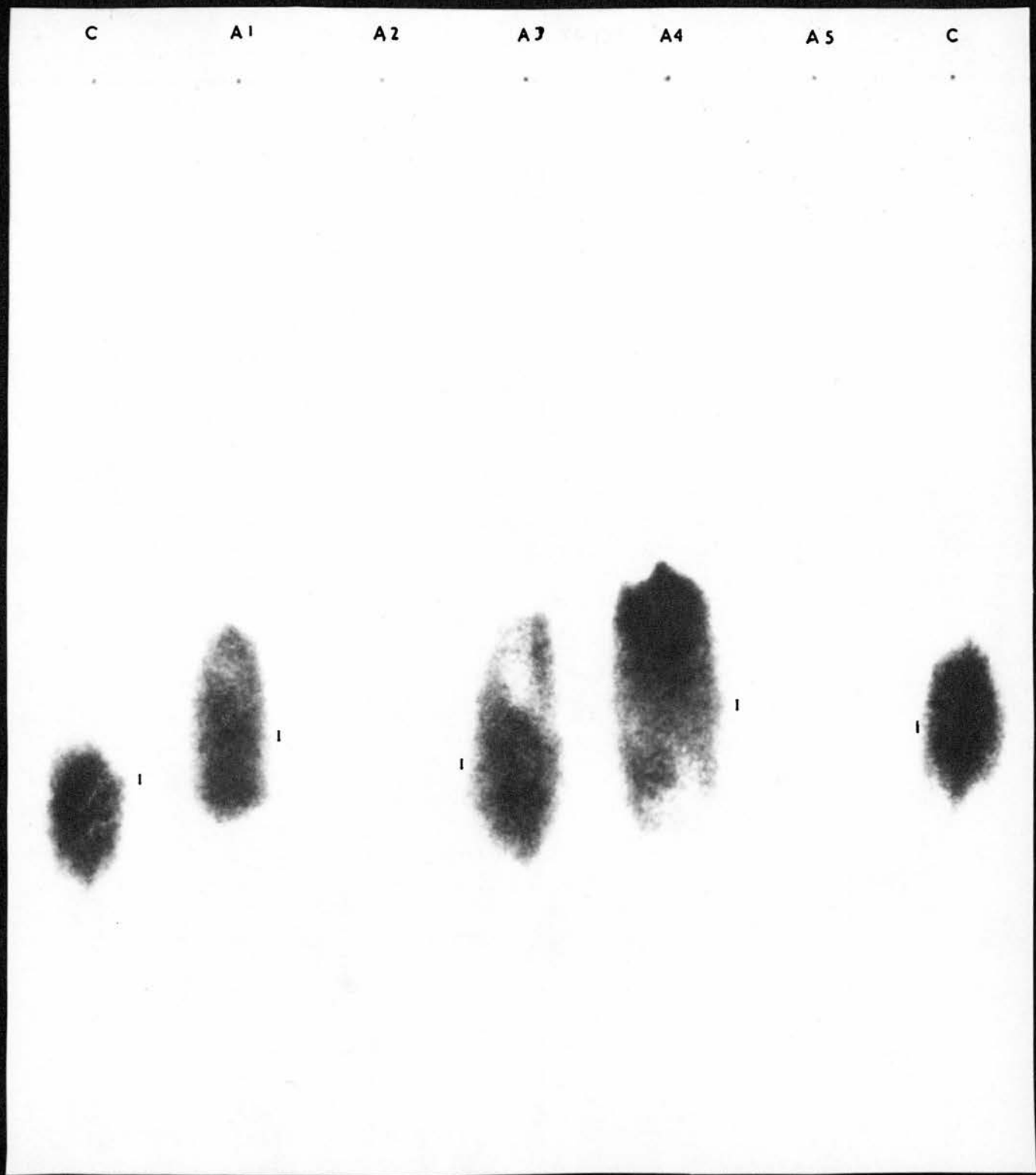

C
A1
A2
A3
A4
A5
C

PLATE V11.

Chromatogram to show the production of disaccharides (eluted from column by 7.5% ethanol) by the action on β-glucosan of enzyme preparations from different sources (at 6 and 29 hr.).

(Solvent, butanol: acetic acid: water).

C. - control.

A1. - reaction with green-malt preparation.

A2. - reaction with finished malt preparation.

A3. - reaction with maize preparation.

A4. - reaction with raw barley preparation.

A5. - reaction with inactivated barley preparation.

1. - cellotriose.

2. - laminaritriose.

3. - cellobiose.

4. - laminaribiose.

5. - trisaccharide(s) possessing β-1,3- and β-1,4-linkages.

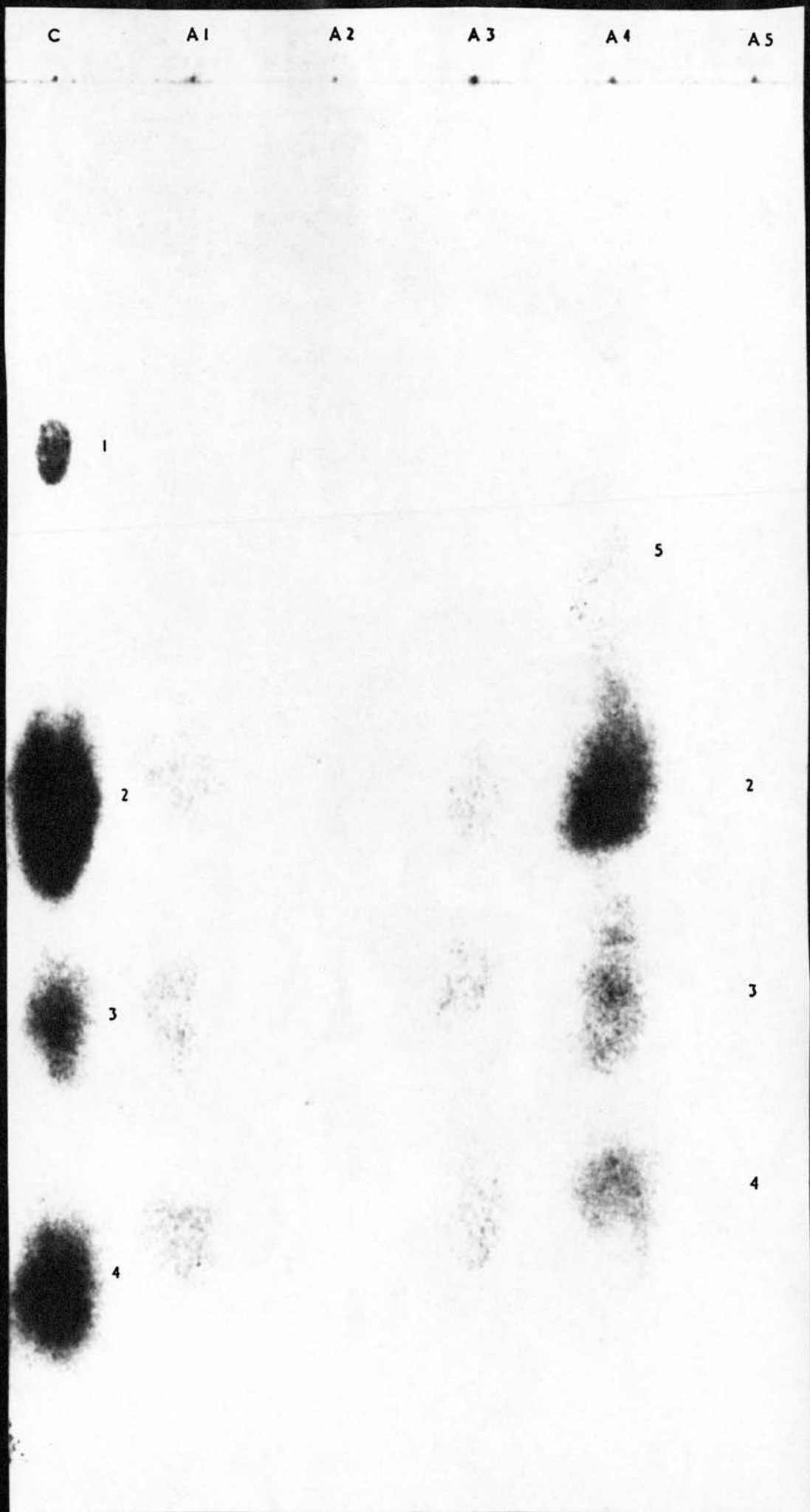

C
A1
A2
A3
A4
A5
1
2
3
4
5
2
3
4

PLATE V111.

Chromatogram to show the production of oligosaccharides (eluted from column by 30% ethanol) by the action on β-glucosan of enzyme preparations from different sources (at 2,6 and 29 hr.).

(Solvent, butanol: ethanol: ammonia: water).

C. - control.

A1. - reaction with green-malt preparation.

A2. - reaction with finished malt preparation.

A3. - reaction with maize preparation.

A4. - reaction with raw barley preparation.

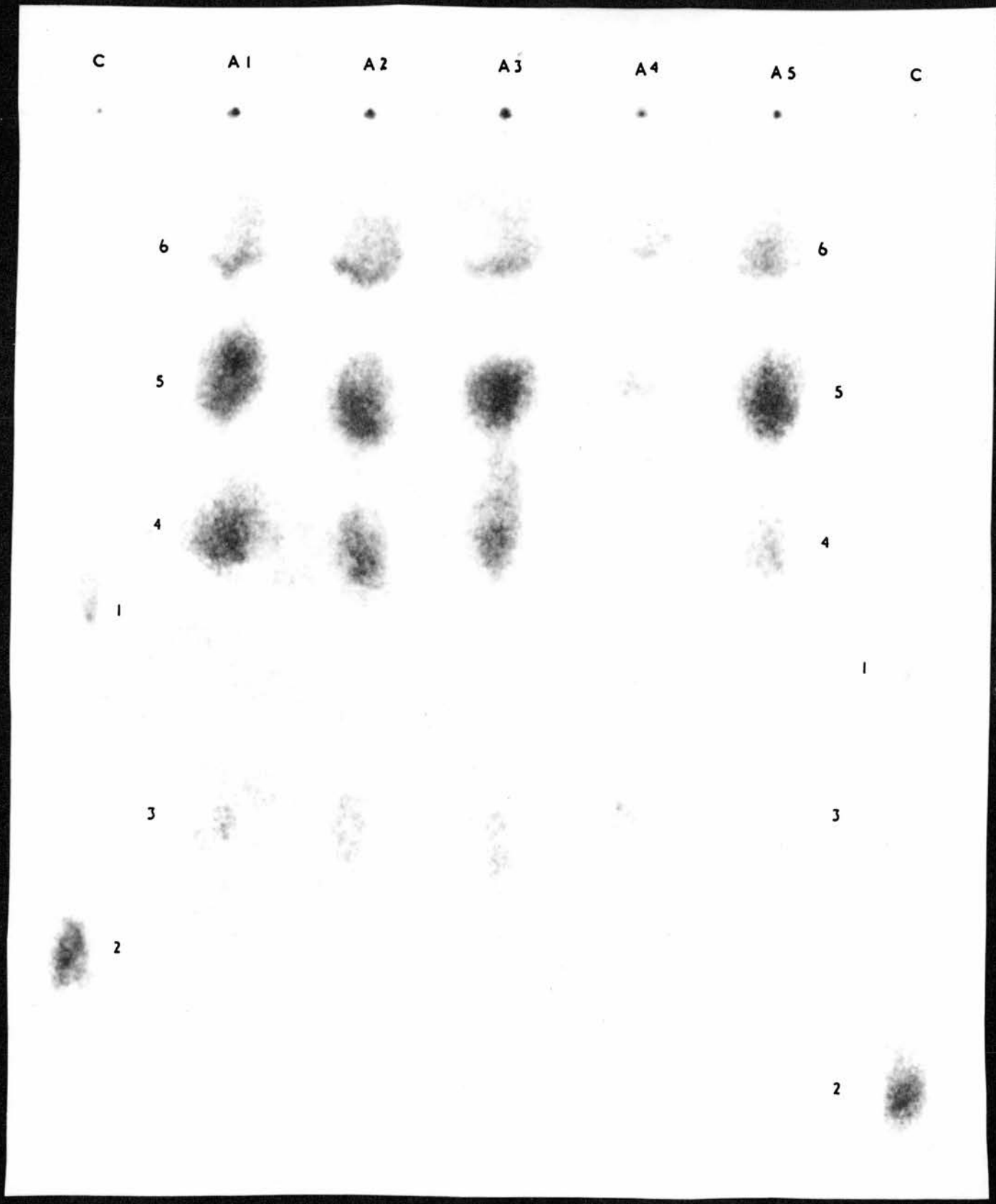
A5. - reaction with inactivated barley preparation.

1. - cellotriose.

2. - laminaritriose.

3. - trisaccharide(s) possessing β-1,3- and β-1,4- linkages.

4,5,6. - oligosaccharides of unknown molecular size.


C
A1
A2
A3
A4
A5
C
6
5
4
1
3
2
6
5
4
1
3
2

and laminaribiose are produced in large amounts by the action of the enzyme preparation from raw barley and only in comparatively small quantity in those reaction mixtures employing maize and green-malt preparations. Disaccharides could not be detected as a result of the action of the finished malt and inactivated barley preparations. A striking feature is the presence of laminaritriose in the reaction mixtures employing raw barley, maize and green-malt preparations. Besides laminaritriose there is also a suggestion of a trisaccharide possessing an R_P value intermediate between those of cellotriose and laminaritriose. Oligosaccharides could be detected after 2 hr. only with great difficulty, the production of these appearing to be maximal at 29 hr., although the patterns at 2, 6 and 29 hr. were similar (Plate VIII). All reaction mixtures produced appreciable quantities of oligosaccharides with the exception of that employing raw barley enzymes.

Enzymolysis of laminarin and β-glucosan "dextrin."

The dextrins produced by the action of green-malt and finished malt enzymes on β-glucosan in the above experiments were subjected to further attack by these preparations. 0.5% (w/v) solutions of the dextrins, buffered at pH 5.0, were treated with their respective enzyme solutions (at concentrations sufficient to decrease the specific viscosity of a substrate having an initial value of 18.1θ to one-half in 10 minutes, each reaction mixture being comparable with respect to endo-activity) at 25°C. for 18 hr. The reactions were terminated by adding four volumes of 95% (v/v) ethanol, the supernatant liquid being evaporated to a very small volume.

Similar experiments were carried out with laminarin as substrate, this being the only variable, substrate concentration, enzyme concentration, temperature and time of reaction being similar /

similar to those employed with the dextrins. When ethanol was added to the reaction mixtures to terminate the reactions, only a very slight precipitate was obtained from the reaction mixture employing the green-malt preparation in comparison with the precipitate obtained from the mixture employing the enzymes of finished malt.

The supernatant liquors, after concentration to a small volume, were applied to chromatograms which were developed for six days by the upper phase of the system butanol:acetic acid:water (40:10:50). Plates IX and X show the results of the action of the green-malt and finished malt preparations respectively on the dextrins and laminarin. It appeared that the action of both preparations on the dextrins was very slight (only negligible amounts of sugars produced) whilst laminarin appeared to have undergone extensive degradation. As a result of their action on the dextrins both preparations produced a trisaccharide possessing the R_F value 0.050, which is intermediate between the values for cellotriose (0.033) and laminaritriose (0.068), an indication that this trisaccharide possesses both a β-1,3- and a β-1,4- linkage (see Plates VII and VIII). At least two higher oligosaccharides are observed and these are the subject of further investigation (vide infra). The green-malt preparation differed from finished malt preparation in producing a trace of laminaritriose from the dextrin.

The actions of both preparations on laminarin produced similar patterns, oligosaccharides up to laminari-octaose being detected. It is interesting to note that these oligosaccharides produced a straight-line graph (Figure 18) when $\log_{10}\frac{R_F}{1-R_F}$ was plotted against molecular size. French and Wild (1953) have shown that when a straight-line /

PLATE IX.

Chromatogram to show the products of the enzymolysis of β-glucosan dextrin and laminarin by a green-malt preparation.

PLATE X.

Chromatogram to show the products of the enzymolysis of β-glucosan dextrin and laminarin by a finished malt preparation.

(Solvent, butanol: acetic acid: water).

C. - control.

L. - enzymolysis products of laminarin.

D. - enzymolysis products of β-glucosan dextrin.

1. - cellotriose.
2. - cellobiose.
3. - laminaribiose.
4. - laminaritriose.
5. - laminaritetraose.
6. - laminaripentaose.
7. - laminarihexaose.
8. - laminariheptaose.
9. - laminarioctaose.
10. - higher homologues.
11. - trisaccharide(s) with β-1,3- and β-1,4-linkages.
12,13,14. - oligosaccharides of unknown molecular size (see later).

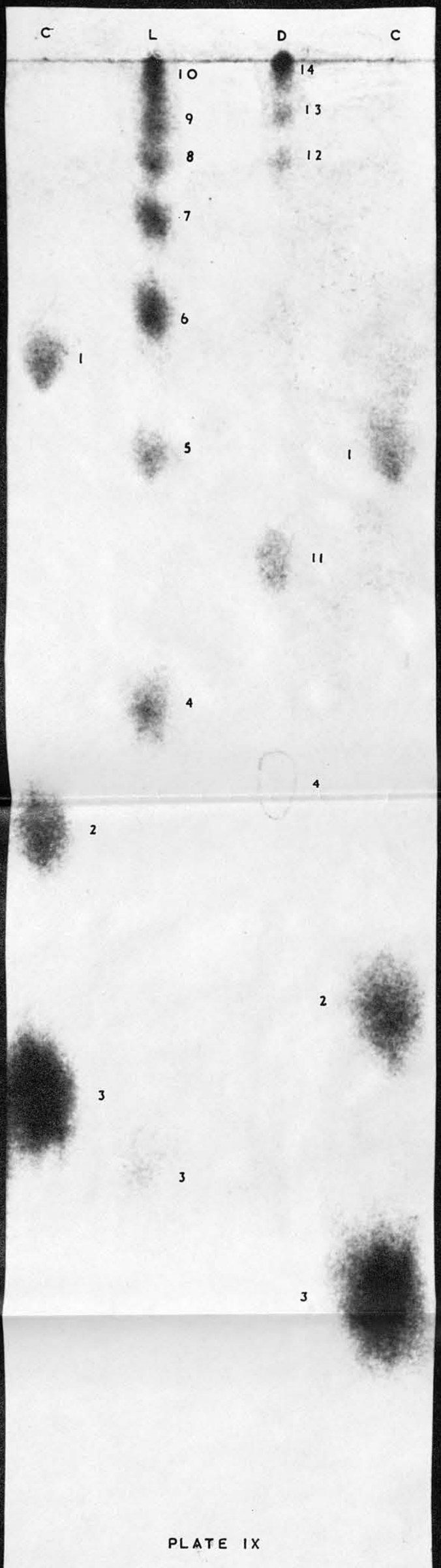


PLATE IX

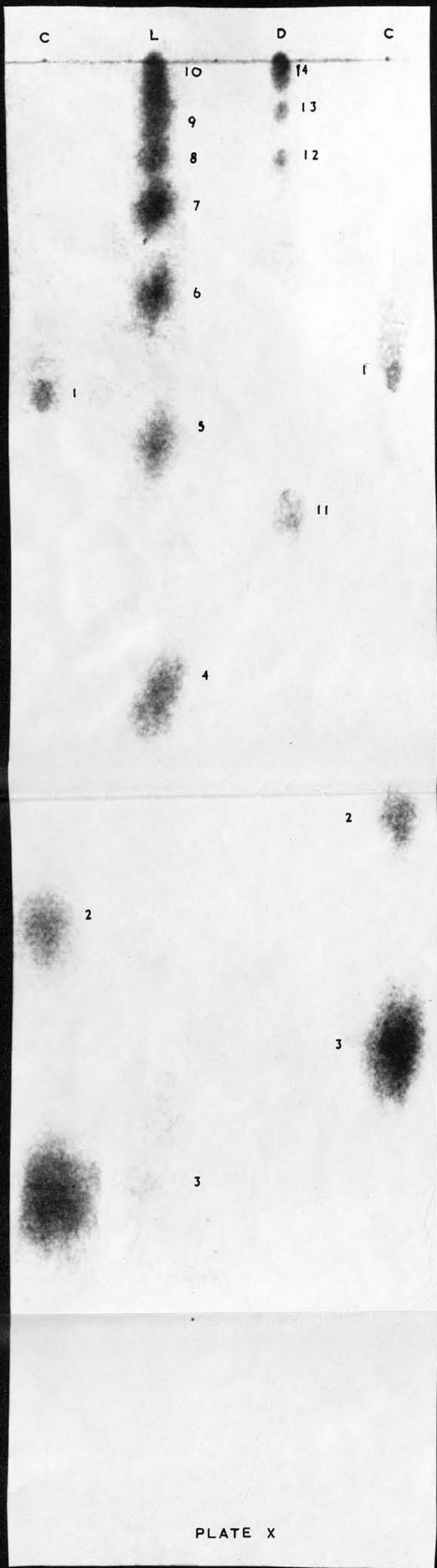


PLATE X

PLATE X1.

Ionophoresis of oligosaccharides as borate complexes at 1000 volts and 5-7 ma. (3 hr.).

C. - controls.

1. - glucose.

2. - cellobiose.

3. - laminaribiose.

4. - laminaritriose.

A - trisaccharide fraction (3 on Plate V111; 11 on Plates 1X and X).

5. - isomer possessing β-1,4-linkage at reducing end

5a. - isomer possessing β-1,3-linkage at reducing end

PLATE X11.

Ionophoresis of oligosaccharides as borate complexes at 1000 volts and 5-9 ma. (3½ hr.).

R. - controls.

1. - laminaritriose.

2. - cellobiose.

A. - oligosaccharide fraction (5 on Plate V111; 13 on Plates 1X & X).

3,3a. - isomers possessing β-1,4- and β-1,3-linkages at reducing ends respectively.

B. - oligosaccharide fraction (4 on Plate V111; 12 on Plates 1X & X).

4,4a. - isomers possessing β-1,4- and β-1,3-linkages at reducing ends respectively.

C. - trisaccharide fraction (A on Plate X1).

5,5a. - isomers possessing β-1,4-and β-1,3-linkages at reducing ends respectively.

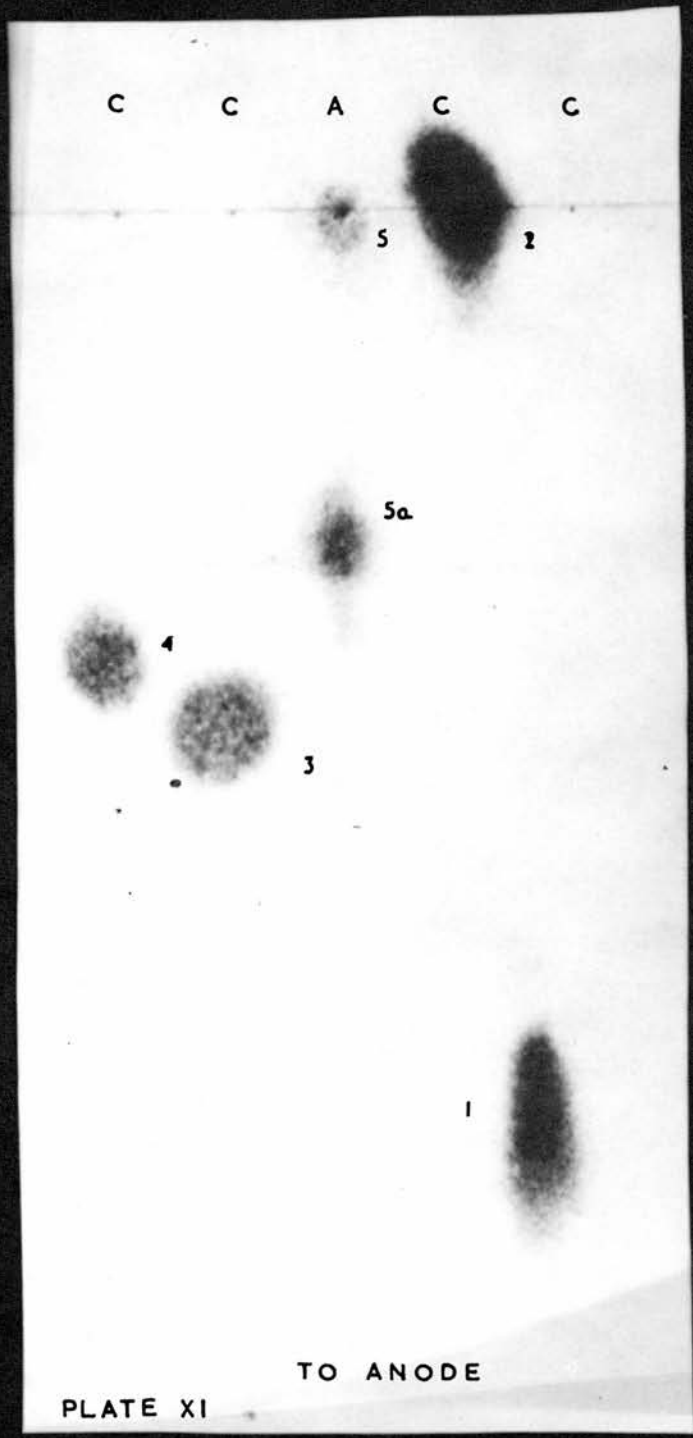


PLATE XI

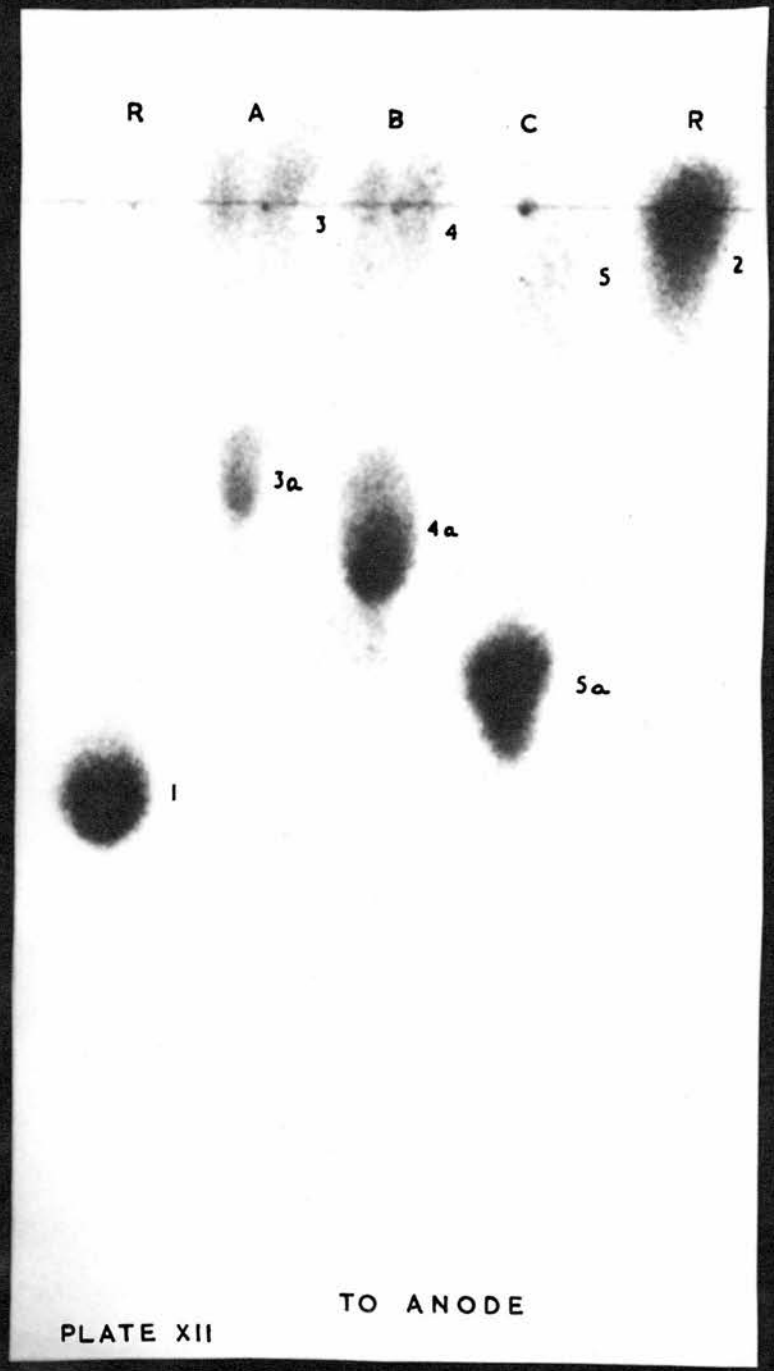


PLATE XII

PLATE XIII.

Ionophoresis of oligosaccharides as bisulphite complexes at 600 volts and 18-31 ma. (4½ hr.).

R. - control.

1. - laminaribiose.

A. - oligosaccharide fraction (A on Plate XII).

4. - Distance migrated suggests a hexasaccharide.

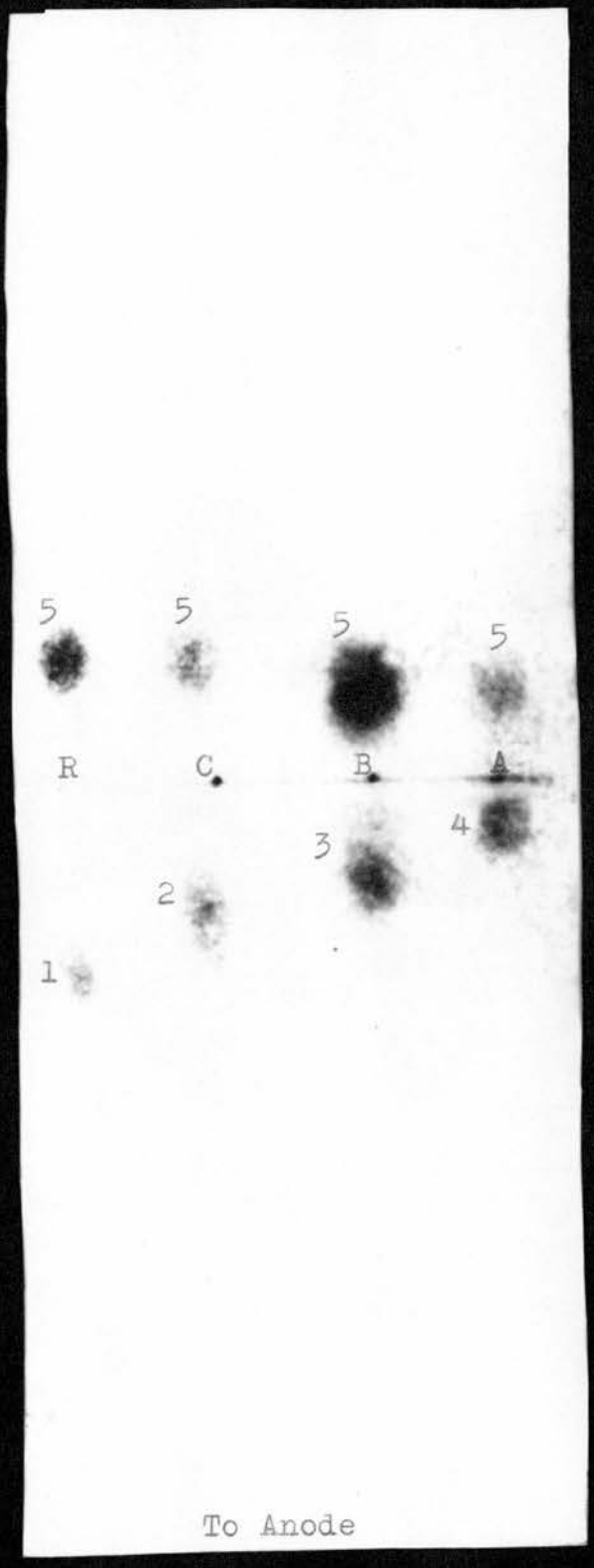
B. - oligosaccharide fraction (B on Plate XII).

3. - Distance migrated suggests a tetrasaccharide.

C. - trisaccharide fraction (C on Plate XII).

2. - Distance migrated proves it to be a trisaccharide.

5. - uncharged oligosaccharides.


5
5
5
5
R
C
B
A
4
3
2
1
To Anode

straight-line relationship is obtained, the oligosaccharides concerned belong to a homologous series.

Ionophoretic analyses.

The trisaccharide (R_F = 0.050) and the two higher oligosaccharides shown in Plates VII-X were eluted separately according to MacLeod (1951). The concentrates of these eluates were subsequently analysed ionophoretically by the method of Foster (1953), in which the electrolyte is 0.2M sodium borate. Plate XI shows the result of ionophoresis of the trisaccharide fraction at 1,000 volts and 5-7 milli-amps for 3 hr. The true M_G values of the different molecules cannot be determined because there is an ever-present electroendosmotic flow. However, Foster (loc. cit.) states that the mobility of an oligosaccharide will be governed largely by the point of attachment of the remainder of the molecule to the reducing moiety. Therefore, from the observed mobilities and with particular reference to laminaritriose, the trisaccharide must have the structure Glβ4Glβ3G, but the isomeric trisaccharide, Glβ3Glβ4G, theoretically possible is only present in very small amount in comparison (these trisaccharides are not separable by ordinary chromatographic methods), and it is seen that the mobility is governed by the presence of a β-1,4- linkage at the reducing end. Plate XII shows the results of a similar analysis but applied to the two higher oligosaccharides, ionophoresis being extended to 3½ hr. at 1,000 volts and 5-9 milli-amps. It will be observed that both oligosaccharides have separated to give two isomers but in those cases it cannot be said that one isomer is more abundant than the other. The distribution of the linkages cannot be shown in those cases; it can only be said that each isomer must have either a β-1,4- or a β-1,3- linkage at the reducing /

reducing end, β-1,3- if it is highly mobile, β-1,4- if it is not.

Employing the method of Frahn and Mills (1956) the molecular sizes of the oligosaccharides were determined. An equal volume of 0.4M sodium bisulphite solution was mixed with the test solution and the mixture was allowed to stand for ½ hr. during which time the bisulphite formed an ionised complex with the oligosaccharide. After applying to Whatman No. 1 paper, and using 0.4M sodium bisulphite as electrolyte, ionophoresis was carried out for 4½ hr. at 600 volts and 18-31 milli-amps. The paper, after drying, was dipped into a solution of aniline picrate in acetone and heated at 100°C. From Plate XIII it is observed that there are two spots for each component. One spot represents electroendosmotic flow of uncharged sugar, whereas the other represents migration of the ionised complex toward the anode. The distance between the spots is a measure of the true ionic mobility of the charged complex. The M_G values decrease regularly (not linearly) with increase in molecular size of the aldose. From the experimental values given by Frahn and Mills (loc. cit.) the equation

$$(M_G + 7)(M.W. + 232.5) = 48665$$

has been calculated, where M_G is the true mobility of the aldose and M.W. is the molecular weight. The graph obtained by plotting M.W. against the corresponding M_G value obtained from this equation is shown in Figure 20. This figure also includes the straight-line graph obtained when M.W. is plotted against the reciprocals of the experimental figures obtained by these workers. The values obtained for the trisaccharide containing the β-1,3- and β-1,4-linkages is 0.56 whilst those for the oligosaccharides are 0.44 and 0.31.

DISCUSSION /

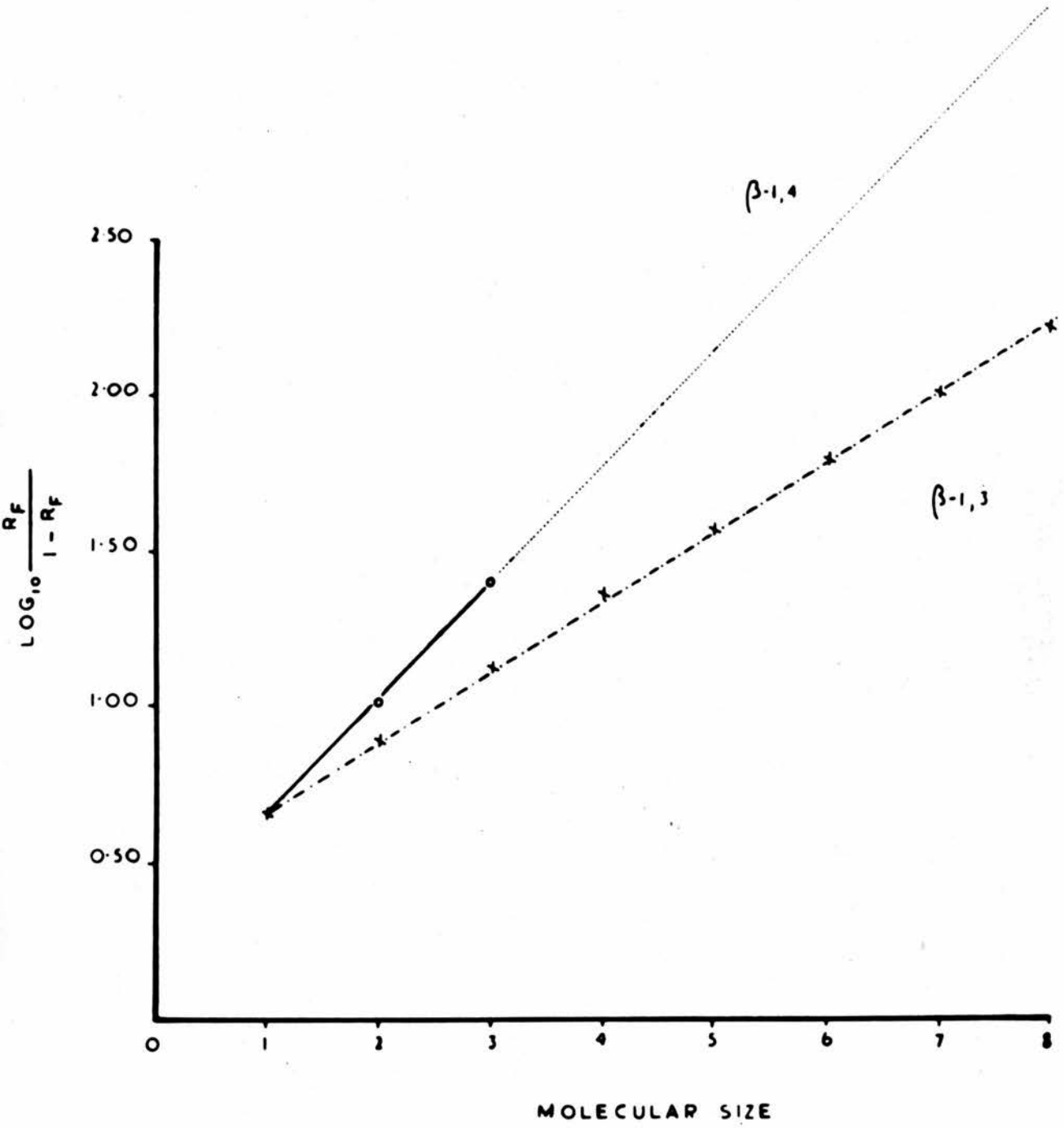


Figure 18.

The straight-line relationships obtained in the respective homologous series of oligosaccharides (β-1,3- and β-1,4-) by plotting $\log_{10}\frac{R_F}{1-R_F}$ against molecular size. The R_F values are those obtained from Plates 1X and X.

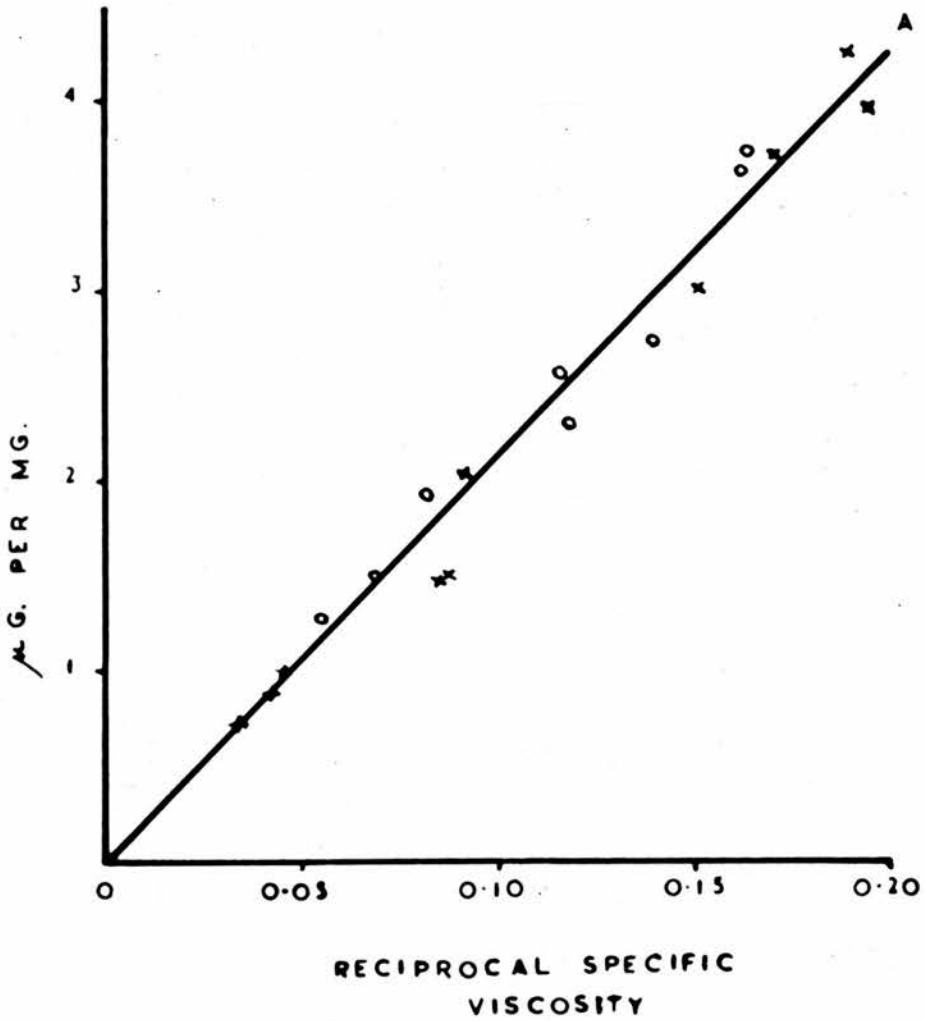


Figure 19.

Correlation between reducing power and reciprocal specific viscosity of β-glucosan samples (o) and between increments of reducing power and change in reciprocal specific viscosity (x) of β-glucosan under the influence of endo-β-glucosanase. The equation to OA is

$$g = 21.19u + 0.001 \pm 0.22.$$

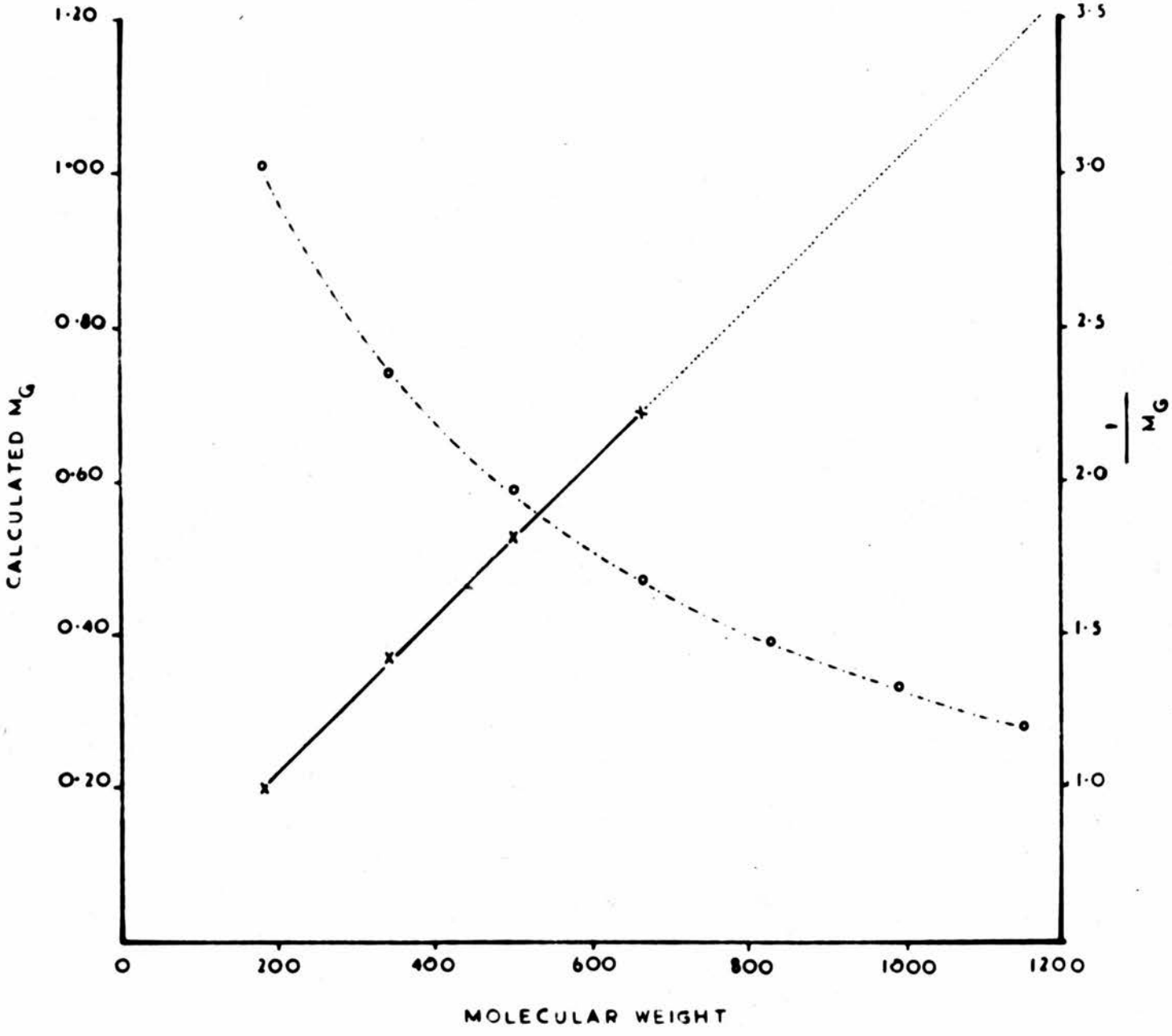


Figure 20.

The straight-line relationship obtained by plotting the reciprocals of the MG values of bisulphite complexes of oligosaccharides in ionophoresis against molecular weight. The curve is the result of plotting the calculated MG values obtained from the equation

$$(M_G + 7)\ (MW + 232.5) = 48665$$

against molecular weight (MW).

TABLE VII.

Influence of pH on Extraction of β-glucosanase Components from Barley.*
(Initial specific viscosities of substrates for Universal Buffer series and the citrate-phosphate buffer series were 7.30 and 13.90 respectively)

pH of Buffer	Citrate-phosphate buffer				Universal Buffer			
	pH of extract	Endo-activity	Exo-activity	Cellobiase activity	pH of extract	Endo-activity	Exo-activity	Cellobiase activity
2.2	3.0	0.0107	0.0100	0.09	-	-	-	-
3.0	3.6	0.0350	0.0173	0.41	-	-	-	-
4.0	4.3	0.0863	0.1050	1.41	5.0	0.0801	0.0934	3.37
5.0	5.1	0.0736	0.1144	1.87	5.5	0.0760	0.0662	2.93
6.0	6.0	0.0446	0.1007	1.55	-	-	-	-
7.0	6.9	0.0306	0.0737	1.39	6.7	0.0655	0.0445	2.81
8.0	7.6	0.0400	0.0798	1.22	7.2	0.0624	0.0371	1.73
9.0	-	-	-	-	7.4	0.0562	0.0322	1.54
10.0	-	-	-	-	7.7	0.0486	0.0159	1.07
11.0	-	-	-	-	8.2	0.0418	0.0130	0.88

* Arbitrary units, and method of calculation as in Section 1.

TABLE VIII.

Influence of pH on Precipitation of β-glucosanase Components from Barley.
(Initial specific viscosity of substrate = 8.47)

pH at which precipitated	Arbitrary units based on standard 11 ml. substrate *		
	Endo-β-glucosanase activity	Exo-β-glucosanase activity	Cellobiase activity
4.0	0.0633	0.1206	-
4.2	0.0778	0.1492	3.20
4.4	0.0960	0.1922	3.61
4.6	-	-	-
4.8	0.0813	0.1685	2.99
5.0	0.0735	0.1612	2.85
5.2	0.0744	0.1780	2.70

* As in Table VII.

TABLE IX.

β-Glucosanase Activities of Enzyme Preparations from Common Cereals.
(Initial specific viscosity of substrate for green-malt and finished malt determinations = 14.49, and for others = 6.17)

Cereal	Arbitrary units based on standard 11 ml. substrate*			
	Yield+	Endo-β-glucosanase activity	Exo-β-glucosanase activity	Cellobiase activity
Oats	31	0.3790	0.0687	0.67
Maize	20	0.1151	0.0071	0.29
Barley	35	0.0980	0.2081	9.84
Wheat	44	0.0603	0.1020	3.36
Rye	45	0.0670	0.3007	0.29
Green malt	-	0.7513	0.2120	-
Finished malt	-	0.9333	0.0000	-

* As in Table VII.

\+ mg. per 100 gm. of cereal.

TABLE X.

Influence of Phenylacetic Acid and of Borate on Maize Enzymes.
(Initial specific viscosity of substrate = 7.30)

Preparation +	Arbitrary units based on standard 11 ml. substrate *		
	Endo-β-glucosanase activity	Exo-β-glucosanase activity	Cellobiase activity
A	0.2517	0.0289	0.52
B	0.2584	0.0024	0.52
C	0.2370	0.0132	0.35

* As in Table VII.

\+ A, control; B, phenylacetic acid; C, borate.

TABLE XI.

Influence of Borate on the β-glucosanase System of Barley.
(Initial specific viscosity of substrate = 6.17)

Conditions of treatment			Arbitrary units based on standard 11 ml. substrate *		
Amount+ of borax	Time (hr.)	Temperature (°C.)	Endo-β-glucosanase activity	Exo-β-glucosanase activity	Cellobiase activity
- φ	-	-	0.0663	0.0668	-
5	6	15	0.0460	0.0319	3.08
10	6	15	0.0460	0.0387	2.75
- φ	-	-	0.0525	0.1158	2.48
5	¼	45	0.0340	0.0468	0.80
5	¼	55	0.0109	0.0000	0.69
5	¼	70	0.0076	0.0000	0.00
5	18	15	0.0566	0.0537	2.39
- φ	-	-	0.0805	0.1447	5.40
5	18	23	0.0703	0.0936	-
5	18	36	0.0265	0.0243	2.97

* As in Table VII.

† mg. per ml.

φ control.

TABLE XII.

Influence of Phenylacetic Acid on the β-glucosanase System of Barley.
(Initial specific viscosity of substrate = 6.17)

Conditions of treatment (15°C.)		Arbitrary units based on standard 11 ml. substrate *		
Amount of reagent †	Time (hr.)	Endo-β-glucosanase activity	Exo-β-glucosanase activity	Cellobiase activity
- φ	-	0.0612	0.1979	5.00
5	½	0.0437	0.0309	3.50
5	1	0.0383	0.0212	4.29
1	2	0.0546	0.1619	3.72
3	2	0.0460	0.0376	3.37
5	2	0.0526	0.0105	1.94
5	4	0.0284	0.0000	1.76
5	5½	0.0288	0.0000	-

* As in Table VII.

† mg. per ml.

φ control.

TABLE XIII.

Influence of Further Physical and Chemical Treatments on Barley β-glucosanases.

(Initial specific viscosities of substrates = 7.04[+], 8.61[φ] and 18.10[ǂ])

Reagent		Conditions of treatment		Arbitrary units *	
Nature	Amount (mg. per ml.)	Time (hr.)	Temperature (°C.)	Endo-β-glucosanase activity	Exo-β-glucosanase activity
None; control [+]	-	-	-	0.0944	0.2031
Acetic acid (pH 3.4)[+]	-	18	15	0.0881	0.0617
None [+]	-	¼	70	0.0052	0.0000
Calcium acetate [+]	2	¼	70	0.0143	0.0000
None; control [φ]	-	-	-	0.0567	0.0837
Iodoacetic acid [φ]	5	3	15	0.0240	0.0519
Arsenic acid [φ]	5	3	15	0.0133	0.0000
Phenylmercuric nitrate [φ]	5	3	15	0.0497	0.0000
None; control [ǂ]	-	-	-	0.0242	0.0532
Papain [ǂ]	0.25	4	15	0.0212	0.0552

* As in Table VII.

TABLE XIV.

Characters of Some β-Glucosan Preparations.

Sample No.	Reciprocal sp. viscosity (0.5% solution; 25°C.)	Reducing power (μg. glucose equivalent per mg.)
1	0.164	3.71
2	0.162	3.62
3	0.140	2.71
4	0.118	2.30
5	0.116	2.57
6	0.072	1.92
7	0.069	1.50
8	0.055	1.28

TABLE XV.

Increases in Reciprocal Specific Viscosity and Corresponding Increases in Reducing Power during Endo-β-Glucosanase Conversions.

Conversion No.	Increase per hr. in	
	Reciprocal sp. viscosity (0.5% solution; 25 C.)	Reducing power (μg. glucose equivalent per mg. of substrate)
1	0.195	3.93
2	0.189	4.24
3	0.170	3.71
4	0.152	3.00
5	0.091	2.02
6	0.086	1.49
7	0.085	1.47
8	0.045	1.00
9	0.043	0.89
10	0.033	0.73

TABLE XVI.

Oligosaccharide Production from β-Glucosan †.

Elution series	Fraction	Enzyme source				
		Raw barley	Barley; treated with phenylacetic acid	Green malt	Finished malt	Maize
Water	Glucose	++++	-	+	-	++
7.5% aq. ethanol	Laminaribiose	+++	-	+	-	+
	Cellobiose	+++	-	+	-	+
	Trisaccharides	++++	-	+	-	+
30% aq. ethanol	Higher oligosaccharides *	++	+++	+++	+++	+++

* Representing at least four oligosaccharides; the distribution pattern is the same, though not in relative intensity.

† Distribution pattern similar at 2, 6 and 29 hr.

DISCUSSION.

Buffer extraction of barley, allied to ammonium sulphate fractionation, has resulted in preparations of enhanced endo-β-glucosanase activity, but it is seen that there is a sharper drop in the exo-activity of preparations obtained by extraction with Universal Buffer (Figure 14) whilst, at the same time, the values for endo-activity are perfectly comparable. The inactivating constituent in Universal Buffer was found to be phenylacetic acid and this led to a series of experiments in which the inactivating effect was examined. The effective concentration was fixed at 5 mg. per ml. and the time, for complete inactivation of exo-β-glucosanase, at 4 hr. Only an examination of Figure 16 reveals the very rapid inactivation of exo-activity and the slow, partial inactivation of endo-activity (approximately 50%) which remains more or less constant after 4 hr. This elimination of exo-activity encouraged work with other reagents with a view to eliminating endo- and cellobiase activities, but it was found that the exo-enzyme was the most susceptible to physical and chemical treatments. Phenylmercuric nitrate effected complete elimination of exo-action whilst retaining 90% of the original endo-activity (Table XIII), being a more efficient reagent than phenylacetic acid, and heat and hydrogen ions also eliminated exo-activity but at the great expense of endo-β-glucosanase activity. The only method which has given an indication that inactivation of endo-activity may be possible is that employing papain according to Meredith et. al. (1953, 1955) when a 13% decrease in endo-activity, accompanied by a slight increase in exo-activity, took place (Table XIII). Further inactivation may be effected by increasing the papain concentration and time of contact of this preparation with the test solution /

solution of enzymes. Inhibition of cellobiase activity by glucono -1:4- lactone is not successful (Plate V).

Although no separation of the three activities has been attained, the threefold nature of the β-glucosanase system has been established. A combination of buffer extraction and ammonium sulphate precipitation at the respective optima has resulted in a more reliable survey of the enzyme potentialities of the five raw cereals. It will be noticed (Table IX) that, contrary to the findings of Preece et al. (1954), maize possesses a small, but significant, cellobiase activity. The oat preparation was shown to have by far the greatest endo-activity with very low exo- and cellobiase activities. In this way the oat preparation resembles the β-glucosanase system of maize which has a lower endo-activity. The barley enzyme preparation possesses a slightly lower cytoclastic activity than maize, but differs greatly from the latter with respect to exo- and cellobiase activities. Wheat presents no special features, whereas the preparation from rye possesses the greatest exo-activity of the series whilst the endo- and cellobiase activities are quite low. These results indicate that measurement of exo-activity by the present Somogyi (1945) method will involve the greatest error when applied to reactions involving the barley enzyme preparation because of the large concomitant cellobiase activity. Included in Table IX are the activity values for green-malt and finished malt preparations. These preparations were originally obtained by the method shown in Section 1 but were re-dissolved in water and precipitated by ammonium sulphate. Green-malt resembles oats in that it possesses a very high endo-activity accompanied by a correspondingly low exo-activity, whilst the finished malt preparation is devoid of exo-

action /

action. The greater endo-activity of the latter preparation over that of green malt is understandable since the concomitants associated with the finished malt preparation will be less complex and, hence, more easily removed during purification.

Plate IV, showing the action of the enzyme preparations from the five raw cereals on cellobiose, confirms what has just been said, but at the same time there is evidence, in all instances, that synthesis has occurred during the hydrolysis. In all reactions a sugar, corresponding to the position of gentiobiose, is observed, whilst another sugar, corresponding to the position of laminaribiose, is observed in all instances with the exception of the oats reaction. These observations are in accord with work published by, among others, Crook and Stone (1957), who were studying the hydrolysis of cellobiose by enzyme preparations from Aspergillus niger. These workers employed reaction mixtures which favoured a high rate of reaction, the cellobiose being present at a concentration of 5% (w/v) and the reactions were conducted at 40°C. They attribute the syntheses of the disaccharides to enzymes which possess both a hydrolytic and a transfer activity. It is possible, then, that the cereal enzyme systems possess similar potentialities.

Measurement of endo-activity.

The results obtained in the present section and, in part, in the investigation of the behaviour of the β-glucosanase system during malting make it possible to examine the original assumption made by Preece, Aitken and Dick (1954) which has formed the basis of the method for calculating endo-activity in the present work. It is assumed that decrease of the viscosity of a β-glucosan solution to one-half its original value involves doubling the initial /

initial reducing power of the substrate when endo-activity alone is involved.

In the first instance, in Table XIV are shown the reciprocal specific viscosities (u; in 0.5% aqueous solutions at 25°C.) and the reducing powers (g; μg. glucose equivalent per mg.) of eight preparations of β-glucosan employed throughout the present investigation. It was found that these values satisfy the relation

$$g = 20.58u + 0.168 \pm 0.23 \qquad (1)$$

and therefore g and u may be regarded as showing direct proportionality. It follows, therefore, from this direct relationship between viscosity and reducing group production that the viscosity is a measure of the average molecular size of a sample. Similarly, Table XV gives the changes in reciprocal specific viscosity (u) and the increases in reducing power (g; μg. glucose equivalent increase per mg. of substrate) during ten β-glucosanase conversions of β-glucosan; the ten enzyme preparations involved in these reactions, selected from results obtained in Section 1 and the present section, had been treated in various ways (by heat treatment, by the action of phenylacetic acid or phenylmercuric nitrate, or were from samples of kilned malt).

These results were found to satisfy the relation

$$g = 21.43u - 0.086 \pm 0.15 \qquad (2)$$

In the same way, increase in reducing power may be taken to be directly proportional to increase in reciprocal specific viscosity. Furthermore, it can be demonstrated that estimates of g for a given value of u do not differ significantly for the two equations; this is what would be expected if the two equations represented findings from two sets of results taken from the same population.

It /

It appears quite in order, therefore, to combine the two sets of results, when the calculated relation becomes

$$g = 21.19u + 0.001 \pm 0.22 \qquad (3)$$

Conversion of this to the working unit, the standard 11-ml. substrate, employed in the present work produces the relation

$$g_m = 1.165u + 0.000 \pm 0.012 \qquad (4)$$

where g_m is the total glucose equivalent (mg.) of the solution ($\equiv$ 55 mg. of β-glucosan).

It can now be stated that enzyme preparations are available which satisfy the basic assumption that to halve the viscosity involves doubling the reducing power, and this can now be demonstrated with a very high degree of accuracy by employing these enzyme preparations.

Arising from the fact that initial substrate characters and enzymolysis changes involving the preparations of Table XV both satisfy so closely the same relation, as shown in Figure 19, and (if the small intercepts are disregarded) independently of the initial specific viscosity of the substrate, a number of important conclusions follow:

(a) In these conversions where exo-activity is absent enzymic hydrolysis of β-glucosan, shown by Aspinall and Telfer (1954) to be a straight-chain molecule, must take place at linkages remote from the chain ends, and therefore true endo-action is involved (this consolidates the original assumption made in 1954 by Preece et al.); this is consistent with the chromatographic evidence put forward by Bass and Meredith (1955) that oligosaccharides are produced by successive degradations of larger molecules and not by random attack. The present work (vide infra) also includes chromatographic evidence in support of this statement.

(b) /

(b) The direct proportionality existing between the liberation of reducing groups and the production of new chain ends, and also the change in reciprocal specific viscosity (equation 2), suggests that the initial viscosity of the substrate - the present figures, of course, can only refer to a substrate possessing an initial value within the working range of 6.17-18.10 - is of no importance of itself in measurements of the activity of endo-β-glucosanase. This supports the empirical evidence put forward by Preece and Aitken (1953), although they were concerned with a narrower range of substrate viscosities (3.47-5.18), but is subject to the proviso mentioned below.

(c) Present evidence justifies the assumption that to halve the viscosity in the early stages of reaction (at least, as shown in Figure 17, until a specific viscosity in the region of 2 has been reached) involves doubling the reducing power.

(d) Where the action of certain β-glucosanase preparations gives rise to anomalies in the sense that the value for reducing group production exceeds that calculated for the observed change in viscosity, then this is clear evidence of a second type of reaction which may be ascribed to exo-activity allied to the action of cellobiase; exo-β-glucosanase activities have, until now, been calculated according to the method shown in Section 1, this being based on the original assumption. If the action of the preparation does satisfy the relation established, exo-activity is absent, and it will be seen from chromatographic evidence (vide infra) that cellobiase action cannot occur during the action of endo-β-glucosanase alone. The present work (Plate VII) suggests that, even at the limit of viscosity diminution (29 hr.), a finished malt preparation (devoid of exo-activity) and a raw barley preparation which /

which had been treated with phenylacetic acid to eliminate exo-activity failed to produce cellobiose and laminaribiose.

Conversion 1 of Table XV has a total reducing power (initial reducing power of substrate + increase during degradation of substrate) of 5.74 μg. glucose equivalent per mg. of substrate corresponding to less than 1% of the substrate weight. It cannot be stated definitely at present what extent of degradation must occur before the relation breaks down. However, it has been observed in protracted conversions that linearity between the reciprocal specific viscosity and time ceases when $\underline{u}$ exceeds about 0.65 (specific viscosity 1.54), as shown in Figure 17, and it may be that this level, which represents a total reducing power of 1.38% calculated on the substrate, is close to the general inflection. It may be advisable, therefore, until further evidence is available, to fix the 1% level as the upper safety limit in making comparative measurements of endo-activity. The ranges of values indicated in Tables XIV and XV have proved quite convenient for working, time of reaction and enzyme concentration being so adjusted in reductiometric experiments as to give titres from 1-5 ml. of 0.005-N sodium thiosulphate solution.

It follows from what has been said that the limiting reciprocal specific viscosities and the limiting reducing powers to be considered when measuring activity are not merely the changes which occur but the final values reached. In other words, it is important to consider the final molecular size attained which means, of course, that the enzymolysis of a substrate possessing a high initial specific viscosity can more safely be studied with higher effective enzyme concentrations or longer times of action than can one of initially lower-molecular size (a substrate with an initial specific /

specific viscosity of approximately 18 is convenient, though it does present certain working difficulties).

Exo-β-glucosanase activity.

It has been shown on the basis of rigid experimental evidence that the direct result of the action of the enzyme or enzyme system endo-β-glucosanase in the earliest stages is the production of new reducing groups proportional to the concomitant increase in reciprocal specific viscosity, and now it remains to consider the criteria available for recognising exo-β-glucosanase activity. These would appear to be (a) the immediate appearance of either or both cellobiose and laminaribiose, without the intervention of higher oligosaccharides; (b) later in the reaction (prior to reaching the inflection shown in Figure 17) the production of disaccharides accompanied by oligosaccharides (Plate VII); and (c) the liberation of reducing groups in excess of those calculated from viscosity change (discussed in (d) above). Since β-glycosidase seems invariably to be present, though from some sources in very small amount, glucose will always accompany the cellobiose and/or the laminaribiose (Plate VI), and whilst β-glycosidase action will render the detection of disaccharides more difficult, the production of glucose will enhance the proportion of reducing groups and therefore the observed excess will be magnified. There is no evidence in the present work for the production of glucose other than by β-glycosidase action. It is pointed out, therefore, than an excess of reducing groups does not afford a measure of exo-action as such, but is merely a demonstration that this action occurs.

In conclusion it is emphasised that precise measurement of exo-β-glucosanase activity from cereal sources is at present impossible /

impossible, particularly in those reactions employing raw barley enzyme preparations, and it is clear also that very small concentrations of exo-enzyme may on occasion be overlooked using the reduction method, though the chance is diminished in view of the enhancing effect of β-glycosidase. Finally, early β-glycosidase action can only occur in those conversions involving exo-action.

Chromatographic and ionophoretic evidence.

Determination of molecular size of β-glucosan dextrins yielded the results 18 and 22 glucose units for two different preparations. Although the order of these figures is undoubtedly correct, finer determinations cannot be carried out by the methods employed. For instance, the value 22 is the mean of those obtained when the Nelson (loc. cit.) method was standardised by cellobiose and laminaribiose, because reference to the fundamental unit of the polysaccharide is necessary. This is not altogether satisfactory and, therefore, a method which is independent of the nature of the linkages in the molecule must be employed. It can be concluded from this investigation, however, that enzyme action, if any, is extremely slow when the chain-length is of this order.

That this is so has been shown by subjecting dextrins, obtained by the action of green-malt and finished malt enzymes to the limit of viscosity diminution, to further action by overwhelming amounts of their respective enzyme preparations. Over a period of 18 hr. enzymolysis must have been negligible because large quantities of high-molecular material were obtained on the addition of alcohol and, furthermore, the oligosaccharide production in both instances was negligible (Plates IX and X). Therefore, there is a suggestion of a resistant "core" in the β-glucosan molecule, this dextrin having an estimated chain-length in the region of 20 glucose units /

units. It may well be that the resistance of this fragment to enzymic attack is connected in some way with the distribution of the β-1,3- and β-1,4- linkages relative to each other. It was possible to detect a trisaccharide (R_p = 0.050) in both instances, the mobility of which suggested the presence of both a β-1,3- and β-1,4- linkage. This recalls the work of Barker et al. (1952) on the polyglucosan from Aspergillus niger and the subsequent analysis of the trisaccharide obtained on partial, acid hydrolysis by Bayly and Bourne (1953) and Foster (1953). Therefore, theoretically this trisaccharide obtained from the β-glucosan dextrins should be homogeneous chromatographically (as it is) and resolvable into two components in ionophoresis (vide infra). The trisaccharide, laminaritriose, was detected in the reaction mixture employing the green-malt preparation and it will be seen below that this sugar is produced in those reaction mixtures employing a preparation containing exo-β-glucosanase. At least two higher oligosaccharides were detected, in both instances, and these were the subject of further investigation.

In comparison, laminarin of a low degree of polymerisation, when reacted with these preparations under similar conditions yielded high concentrations of oligosaccharides (Plates IX and X). This is further evidence for the presence in green malt and finished malt of an enzyme system capable of splitting β-1,3- linkages very rapidly. It is interesting too that, in the reaction employing the green-malt preparation with laminarin, recoverable material was negligible on the addition of alcohol. This may be taken as a demonstration that green-malt preparations do possess an exo-β-glucosanase whilst finished malt preparations do not, because in the case of the latter a large quantity of recoverable material, in

comparison /

comparison, was obtained. In other words, the cytolytic system of enzymes has effected a greater degradation of laminarin. Oligosaccharide patterns obtained as a result of the actions of these preparations were similar and it was found that a straight-line relationship arose when $\log_{10}\frac{R_F}{1-R_F}$ was plotted against molecular size (Figure 18). According to French and Wild (1953) this indicates that the oligosaccharides are members of a homologous series; in other words, laminarin - or at least that part of the molecule which is now degraded - appears to be a straight-chain molecule (anomalous points would have arisen if branching occurred in the polysaccharide molecule) which consists largely of glucose units linked β-1,3. Manners and Anderson (1957) have obtained evidence consistent with the presence of mannitol in the laminarin molecule and therefore this could account for the small amount of recoverable material in the green-malt reaction.

In the series involving the action of the enzymes of raw barley, inactivated barley (treated with phenylacetic acid), maize, green malt and finished malt on β-glucosan, the striking features were the absence of glucose, cellobiose and laminaribiose from those reaction mixtures employing the finished malt and inactivated barley preparations and the large quantities of these sugars produced by the action of the raw barley preparation in comparison with the amounts detected in the green-malt and maize enzyme reaction mixtures (Plates VI and VII). However, a difficult point to explain is the presence of laminaritriose in those reaction mixtures employing the raw barley, green-malt and maize preparations (Plate VII) and its absence where endo-activity alone is involved. Also present as a result of the action of the raw barley preparation is a trace of a trisaccharide of R_F value intermediate between /

between those of cellotriose and laminaritriose, and thus confirmation has been obtained for its detection in the reaction mixtures employing β-glucosan dextrins as substrate (Plates IX and X). Oligosaccharide production was maximal at 29 hr., probably the result of increased degradation of higher-molecular material as the reaction proceeded, but it is interesting to note that, although the pattern is similar in all instances (Plate VIII), there appears to be a decreased production of oligosaccharides in the raw barley reaction mixture. This is explained on the basis of appreciable action by the comparatively large amount of exo-activity present because whilst there is evidence of a limit polysaccharide for the action of the endo-enzyme, the limit for the exo-component must be very much lower. Plate VIII also indicates that the trisaccharide possessing the β-1,4- and β-1,3- linkages is also present in these fractions from the different reaction mixtures.

The results, which are summarised in Table XVI, are consistent with the initial production by endo-β-glucosanase of higher oligosaccharides, the progressive degradation of these to smaller molecules by the endo-enzyme and also, where present, by the action of the exo-enzyme. As a result of cytolytic action (endo- + exo-) the disaccharides cellobiose and laminaribiose are produced, with glucose formed indirectly by the action of β-glycosidase (the specificity of the β-glycosidases is not known). The oligosaccharide pattern produced by the action on β-glucosan of finished malt and inactivated barley preparations provides support for the earlier conclusion (a) that oligosaccharides are produced by successive degradations of larger molecules as a result of endo-action, and not by random attack, because cellobiose, laminaribiose and /

and glucose cannot be detected in these reaction mixtures. Support is also forthcoming from these results for the criteria put forward for the recognition of exo-activity. The results obtained in the present work are in agreement with the chromatographic patterns presented by Bass and Meredith (1955) and the pattern of degradation suggested also accords with the views of these workers.

Ionophoretic analyses of the oligosaccharides served only to complicate matters. The trisaccharide of R_F = 0.050 was resolved into components by the method of Foster (loc. cit.), as shown in Plate XI, which was conclusive evidence for the presence of a β-1,4- and β-1,3- linkage therein. However, it is curious that the isomer of structure Glβ4Glβ3G seemed to be more abundant than the other of structure Glβ3Glβ4G, this latter remaining, in accord with Foster's observations, on the starting line. The mobility of the faster-moving component with respect to laminaritriose and also the fact that the R_F value, when treated by the method of French and Wild (loc. cit.), did not coincide with the graphs for the β-1,4- or β-1,3- homologous series (Figure 18) proved the mixed nature of the linkages. Finally, molecular size determinations by the method of Frahn and Mills (loc. cit.) showed that the oligosaccharide was in fact a trisaccharide (Plate XIII and Figure 20).

Application of ionophoresis, both in borate and bisulphite, to the higher oligosaccharides (12 and 13 in Plates IX and X; 4 and 5 in Plate VIII) showed that each could be resolved into a faster- and a slower-moving component indicating the presence of β-1,3- and β-1,4- linkages at the reducing ends of the molecules respectively and, furthermore, they appeared to be a tetrasaccharide (12 in Plates IX and X; 4 in Plate VIII) and probably a hexasaccharide as indicated by measurements from Plate XIII and application to Figure 20 /

20 (13 in Plates IX and X; 5 in Plate VIII). Confirmation of this latter may be necessary, but it is unlikely that greater migration of the bisulphite complex will be obtained by lengthening the time of ionophoresis (mobility is not proportional to time). However, ionophoresis in borate revealed that the isomers in each instance are present in approximately equal quantities. At these levels the distribution of the linkages within the molecules cannot be revealed by these methods, but it may be significant that application of the $\log_{10}\frac{R_F}{1-R_F}$ values to Figure 18 shows that they do not coincide with the graphs for either homologous series. Furthermore, the quantities available prevented the application of any other method.

The interpretation of chromatographic and ionophoretic results to give a picture of the distribution of the linkages within the β-glucosan molecule requires great care. In the first instance, an alternating pattern of β-1,4- β-1,3, etc. would be indicated by the production of two isomers of the trisaccharide (R_F 0.050) just as found in the present work, but it would not be expected to obtain one isomer in greater quantity than the other if this were so. On the other hand, if the linkages were distributed in groups of β-1,3 and β-1,4, as stated by Aitken et al. (1956), then the presence of both cellotriose and laminaritriose would be evident. In the present investigation laminaritriose is obtained in quantity (Plate VII) but there is no evidence for the production of cellotriose from β-glucosan. If, as shown by Aspinall and Telfer (1954), the β-1,4- and β-1,3- linkages are present in equal numbers, and the molecule is a straight-chain glucose polymer, laminaritriose should only be witnessed in the presence of cellotriose. However, it is curious that the laminaritriose is only detected /

detected in those reactions employing preparations possessing exo-activity. Evidence in support of the work of Aspinall and Telfer (loc. cit.) was published recently by Montgomery and Smith (1956), who state that Gilles et al. (1956) have obtained evidence for the alternate distribution of the linkages in β-glucosan. Gilles et al., having determined that the β-1,4- and β-1,3- linkages are present in equal numbers by a periodate oxidation technique, then formed, from the polyaldehyde obtained by periodate treatment, osazones by the action of phenylhydrazine and it was found that only glucose phenylosazone was present. They state that the presence of glucose residues joined by consecutive β-1,3- linkages would be revealed by the formation of osazones of one or more glucose oligosaccharides and not simply by the osazone of the monosaccharide. However, accepting now that the β-1,4- and β-1,3- linkages are present in equal amounts, the present evidence is not consistent with a straightforward, alternate distribution of the linkages.

Whelan (1957) has obtained evidence which suggests that in the lichenins from oats and Iceland moss there is a regular repeating pattern of Glβ 3Glβ 4Glβ 4G, etc. Lichenin is a glucose polymer possessing a ratio of β-1,4: β-1,3- linkages of approximately 2.5:1. Therefore, it may be that, in barley β-glucosan, there is a regular repeating pattern of Glβ 3Glβ 3Glβ 4Glβ 4G, etc. To explain the presence of laminaritriose and the absence of cellotriose this structure would require a selective hydrolysis of β-1,4- linkages.

It will be realised, of course, that no firm conclusions can be forwarded without a more extensive examination of the fragments produced as a result of the degradation of the β-glucosan molecule by the β-glucosanase system of enzymes.

SUMMARY.

Extraction and precipitation of cereal enzymes at the respective optimum pH values yield products of enhanced purity, whilst studies in the differential inactivation of the barley β-glucosanase system of enzymes have revealed the striking inactivation effect of phenylacetic acid upon exo-β-glucosanase (Figure 16).

It has been shown that the β-glucosanase systems of the five raw cereals differ significantly in the relative distribution of the three aspects of activity. During the hydrolysis of cellobiose by these preparations evidence of synthesis arose (Plate IV).

The early assumption that to halve the viscosity of a β-glucosan solution in the early stages of reaction involves doubling the reducing power is now justified on the basis of rigid experimental evidence. Furthermore, chromatographic evidence indicates that oligosaccharides produced from β-glucosan by endo-action arise as a result of successive degradations of larger molecules and not by random attack.

Criteria, reductiometric and chromatographic, have been put forward for the recognition of exo-action.

Evidence suggests that glucose is the result of β-glycosidase action only and that glucose is produced only when the exo-enzyme is present. No method has yet been found of measuring exo-activity free from cellobiase activity.

The threefold nature of the cereal β-glucosanase system has been clearly established.

There is evidence which suggests the presence of a laminarinase in malt and also that at least the greater part of the laminarin molecule is of straight-chain structure.

GENERAL DISCUSSION.

It is the purpose of this section to review the results obtained in the present work and, where relevant, to correlate the results set out in the experimental sections. An additional object of this discussion will be to put forward suggestions for future work. Furthermore, care in the interpretation of certain of the data will be emphasised.

The practical importance of the enzymolysis of the non-starchy polysaccharides during the growth of the barley corn is very great. Therefore, with a view to advancing the knowledge of this complicated process, it was necessary - Preece and Mackenzie (1952) having obtained already a pure, homogeneous substrate - to obtain enzyme preparations of increased purity and, hence, activity. Initially, the present investigation employed the extraction and precipitation methods of Preece et al. (1954) but later, an advance was made by the application of more chemical means of extraction. However, it is now considered that the further study of these methods in the extraction of cereal β-glucosanases, plus ammonium sulphate precipitation at the optimum pH, is no longer of any great importance; it is doubtful if further significant purification could be attained along these lines. The application of preparations extracted under these conditions to treatment by newer, physico-chemical methods such as column chromatography and ionophoresis is of primary importance, because only under these conditions will separation of the active components within the β-glucosanase system be possible. A method of this type has been described recently by MacWilliam and Harris (1957) in which the enzyme preparation is dissolved in citrate-phosphate buffer, followed by adsorption on an alumina column, and the subsequent elution /

elution of the enzymes carried out under an applied pH gradient. Analyses of the fractions indicated that, among other things, a separation of cellobiase and laminaribiase had been achieved. This evidence is of particular significance to the present work because there has been a doubt concerning the specificity of the β-glycosidases, and it was thought that glucose production during the enzymolysis of β-glucosan by, for instance, a raw barley enzyme preparation may have been the result of the action of a single, non-specific β-glycosidase. It can now be affirmed that glucose production is the result of the joint action of cellobiase and laminaribiase upon the respective disaccharides produced during the enzymolysis of β-glucosan. Furthermore, in view of the inherent difficulty in detecting laminaribiose, in comparison with cellobiose, it appears that the β-1,3- linkages are hydrolysed more rapidly.

As a result of this work by MacWilliam and Harris (loc. cit.) it seems that the laminarinase system may also consist of endo- and exo-components. Such a situation would strengthen an opinion forwarded earlier that there is a standard mode of attack by the polysaccharases, because it was noticed that the β-glucosanase system had many points in common with the amylases. Each system is known now to consist of an enzyme capable of effecting most profound changes in the polysaccharide molecule by rupturing internal linkages (α-amylase and endo-β-glucosanase respectively), this action being accompanied by that of a component which splits off disaccharide units from the chain ends (β-amylase and exo-β-glucosanase respectively), whilst the disaccharides are finally hydrolysed by the glycosidases, maltase in starch degradation, cellobiase and laminaribiase being involved in β-glucosan breakdown.

It /

It is highly probable that the enzyme system responsible for the enzymolysis of β-glucosan will also effect a closely similar action upon the related polysaccharide, lichenin.

This threefold nature of the β-glucosanase systems of the raw cereals can be accepted with a great degree of certainty as a result of evidence obtained from independent investigations in the present work. Endo- and exo-enzymes and cellobiase have been shown to produce different patterns of activity during the malting process, the action of specific reagents has distinguished between endo- and exo-β-glucosanases, whilst chromatography of β-glucosan reaction mixtures has demonstrated the difference between those systems possessing the exo-enzyme (plus cellobiase and laminaribiase) and those devoid of this activity. The significance of the statistical analyses has been pointed out already but it is necessary that mention be made here because these analyses were based upon results drawn from the whole investigation. Thus, on the basis of rigid experimental evidence it has been established that exo-activity can be eliminated by the application of appropriate means, and so it has been possible to show that true endo-action is involved when the halving of the viscosity of a β-glucosan solution results in the reducing power being doubled. Various criteria are laid down by which it may be possible to recognise exo-activity, but it is important to note that a major advance has been made in consolidating the original assumption put forward by Preece et al. (1954) because this has formed the basis of the calculation of endo- and exo-activities since that time.

It is now possible to describe the mode of breakdown of initially-water-soluble β-glucosan with some greater degree of certainty than has been possible hitherto. The degradation depends /

depends upon two types of change, cytoclastic (endo-action alone) and cytolytic (endo- plus exo-action). The cytoclastic change involves splitting the initially large molecules to progressively smaller ones, the resultant rapid decrease in molecular size giving a rapid decrease in solution viscosity with comparatively little production of new, end reducing groups. This is the state of affairs in those systems devoid of exo-activity, but where this enzyme is present the cytolytic action contributes to the degradation characteristically. The cytolytic action gives, in the main, a large production of reducing groups and is favoured at the lower levels of molecular size when many new, chain ends are available, at which stage the result of the concomitant endo-action is less noticeable (Figure 17). The possibility of synthesis involving oligosaccharides at various levels of complexity is not to be excluded (in the present work cereal enzyme preparations have been shown to effect a synthesis of oligosaccharides), and the present investigation in the earliest stages of reaction will need to be supplemented by further studies of what happens later. However, until the relative distribution of the β-1,4- and β-1,3- linkages is known, a complete picture of the degradation of β-glucosan cannot be obtained. Work carried out with this intention in mind was hampered by the very small amounts of the oligosaccharides produced; therefore, supplementation of the results obtained by chromatographic and ionophoretic analyses by more direct methods, such as periodate oxidation, was not possible. It was possible to show that the distribution of linkages within the straight-chain molecule of β-glucosan is not quite as simple as suggested by Aitken et al. (1956) and Gilles et al. (1956).

It is suspected that the relative distribution of the linkages may /

may be of great importance in determining the extent to which degradation occurs. For instance, other than this, no reason can be found to explain the resistant nature of the dextrin, obtained at the limit of rapid viscosity diminution, to further attack by the β-glucosanase system. Comparison of this dextrin with the β-glucosan obtained from finished malt is quite interesting because evidence obtained has suggested that this represents the limit of degradation of β-glucosan in the malting process; it appears that degradation of β-glucosan must be very slight in finished malt since the percentages of recoverable material in the initially soluble and insoluble hemicelluloses are approximately the same. With the level of endo-activity known to be present in finished malt failure to recover β-glucosan would be expected if further degradation occurred. Furthermore, if these products are of similar molecular size then the glucosan will make little contribution to wort viscosity in practice. Again, assuming they are similar, in view of the resistant nature of the dextrin to further enzymolysis plus the negligible level of endo-activity detected in wort, it must be concluded that the contribution by β-glucosan to worts obtained by infusion mashing must be very small and must arise from the material already in a soluble form. It can only be assumed that the contribution of pentosans to wort properties is no greater but it is not known to what extent degradation has occurred during malting.

Solubilisation of initially insoluble hemicelluloses is another problem which requires very careful study and equally great care in the interpretation of results. That solubilisation occurs is undoubted; it is the manner in which this takes place that is doubtful. In view of the very high level of endo-activity developed /

developed during growth it would be very simple to postulate a purely enzymic effect, but available evidence suggests that, though this may be an important factor, a mechanical action is also involved. Pentosan solubilisation, on the other hand, would appear to be predominantly the result of a mechanical action alone, in view of the low level of pentosanase activity developed during growth. However, results obtained from *in vitro* experiments again require equally careful interpretation, because it is suggested from an examination of Figure 11 that degradation of pentosans *in vivo* is much greater than one would expect from the pentosanase activities recorded by Preece and MacDougall (1956). However, solubilisation of pentosans may be stated tentatively to be the result of mainly a mechanical effect augmented by a small but significant enzymic action.

As a result of evidence contained herein and that given by Cook (1957), no great value can be placed upon methods which attempt to assess malting quality of a range of barleys from endo-β-glucosanase activities, either in the raw barley or green malt, or from the gum content of the raw barley itself. It has become abundantly clear that too many factors are involved and an excess activity is developed by the grain during growth, the important enzymic effects taking place before the significant increases in activity occur. In the present work very little has arisen to assist the maltster in this respect, but it may be of value to record the ratio of initially insoluble hemicellulose of barley to the finished malt content for a number of samples. It would appear that the higher the ratio the more complete is the modification. This investigation has also shown that autolytic experiments do not predict the significant changes in hemicellulose recovery which /

which occur during the growth of the grain.

Throughout the present work care in the interpretation of results obtained by these *in vitro* studies has been rigidly exercised. We have been confronted with the additional problem that no two samples of barley, whether or not from the same variety, are alike and, where possible, the same sample of barley has been employed in those experiments requiring strict comparison. However, it is considered that the position has been reached at which it is possible to obtain results which suggest patterns of possible change *in vivo* by the use of the homogeneous substrate, barley β-glucosan, and the active β-glucosanase preparations obtained by the methods described. Under these conditions the difficulties in interpreting results are decreased but not entirely eliminated, but some workers still persist in increasing their difficulties by the use of artificial substrates.

Several lines of future work can be seen. The investigation of Preece and MacDougall (1956) into the factors concerned in the solubilisation of initially insoluble hemicelluloses is of great importance but is an extremely difficult problem. The solubilisation of β-glucosan is the one step in the enzymolysis of this material which is not abundantly clear at the moment. However, being now in a position to describe the mode of breakdown of soluble β-glucosan according to the nature of the β-glucosanase system present, it seems necessary to examine more closely the actual products of degradation, that is, the oligosaccharides and the β-glucosan dextrin and to compare this latter with the β-glucosan of finished malt. In this respect modern methods of paper chromatography and ionophoresis may have served their purpose and the application of more direct methods of analysis /

analysis suggest themselves. As has already been said, the position has been reached at which to attempt further purification of the β-glucosanase complex along the present lines would no longer be profitable; the urgent problem is now to subject available preparations to techniques by which separation of distinct systems and, later, the components of these systems, concerned in the degradation of β-glucosan can be effected. Column ionophoresis appears to have great possibilities in this respect. Finally, the further pursuit of means whereby specific inactivation is effected, is also desirable especially with a view to inactivating or inhibiting cellobiase activity.

Although a certain advance has been made in our knowledge of the degradative scheme concerned in the enzymolysis of cereal gums (with particular reference to barley β-glucosan) as a result of the present investigation, like most researches, the complexity of the problem has increased many times. It is hoped, however, that it has opened up new ground for further investigation of this problem of great practical importance. It is regretted that very little has been achieved to assist the practical maltster.

PUBLICATIONS.

Material from Sections 1 and 3 has been published in a paper entitled "Observations on the β-Glucosanase System and its Development during Malting" (J. Inst. Brew., 1956, 62, 486).

The results contained in Section 2 are the subject of a paper entitled "Carbohydrate Modification during Malting", to be read at the European Brewery Convention, Copenhagen.

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